Universidade de Aveiro Departamento de Química

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Daniel Boer Bonifácio

Isolamento endógeno de plasmídeos e avaliação do potencial biotecnológico de bactérias associadas a esponjas marinhas

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia ramo Industrial e Ambiental, sob a orientação científica do Doutor Newton C. Marcial Gomes, Investigador Principal do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro e sob a coorientação científica da Doutora Maria Ângela Sousa Dias Alves Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

Dedico este trabalho aos meus pais.

O júri

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Palavras-chave

Porifera, Esponjas marinhas, Microbioma, Plasmídeos, Potencial Biotecnológico

Resumo

O microbioma das esponjas marinhas representa um tópico de investigação de interesse crescente uma vez que é ainda incipiente o conhecimento sobre as interações ecológicas entre os poríferos e a sua microflora e sobretudo, pelo seu potencial interesse comercial. Um elevado número de substâncias, incluindo moléculas farmacologicamente ativas, foi já identificado em esponjas marinhas e nos seus microrganismos associados. A elevada diversidade do microbioma de esponjas enquanto comunidade microbiana leva à hipótese de que este possa também conter uma grande diversidade de plasmídeos. No entanto, a informação disponível sobre o papel dos plasmídeos (mobiloma) nas interações entre os poríferos e os seus simbiontes ou na produção de metabolitos é ainda escassa. Este trabalho teve como objetivo a exploração do potencial biotecnológico de bactérias associadas a esponjas marinhas através do isolamento de plasmídeos e da caracterização bioquímica das bactérias cultiváveis do microbioma.

O isolamento de bactérias contendo plasmídeos foi feito em meios de cultivo suplementados com antibióticos (cloranfenicol, canamicina, tetraciclina, trimetoprim ou metais/metaloides tóxicos (arsenito de sódio, cloreto de mercúrio) como agentes seletivos. Esta abordagem permitiu obter 42 estirpes das quais 18 continham plasmídeos. O meio seletivo contendo trimetoprim permitiu selecionar o maior número de isolados e o maior número de plasmídeos. No entanto, os meios suplementados com cloranfenicol e canamicina resultaram na maior percentagem de estirpes contendo plasmídeos. Para a caracterização fisiológica e bioquímica dos isolados, avaliou-se a produção de hemolisinas, biosurfactantes aniónicos e inibidores de *quorum sensing*. A atividade hemolítica foi detetada em isolados pertencentes aos géneros *Bacillus*, *Pseudomonas, Psychrobacter*, *Shewanella* e *Vibrio.* Nenhum dos isolados evidenciou produção de biosurfactantes aniónicos. Isolados dos géneros *Pseudomonas, Vibrio* e *Bacillus* mostraram atividade de inibição de *quorum sensing*.

Tendo em consideração os dados obtidos, em trabalhos futuros, pretendemos proceder à sequenciação dos plasmídeos obtidos e à identificação de genes que codificam para vias metabólicas envolvidas na degradação de moléculas específicas, tais como N-Acil homoserina lactonas, e para outras famílias de compostos de interesse biotecnológico.

Keywords Porifera, Marine sponges, Microbiome, Plasmids, Biotechnological Potencial

Abstract

The microbiome of marine sponges represents an emerging research topic since there is a lack of knowledge on the ecological interactions between Porifera and its microflora and there is a great potential commercial interest in bioactive compounds produced by the sponge holobiont. A high number of substances, including pharmacologically active molecules, have already been identified in marine sponges and their associated microorganisms. The high diversity of the sponge microbiome as a microbial community, leads to the hypothesis that it may also represent a large and diverse reservoir of plasmids. However, available information on the role of plasmids (mobilome) in the interactions between Porifera and their symbionts or in the production of metabolites is still scarce. The objective of this work was to explore the biotechnological potential of bacteria associated with marine sponges through the isolation of plasmids and the biochemical characterization of the host bacteria.

Isolation of plasmid-containing bacteria was done in culture media supplemented with antibiotics (chloramphenicol, kanamycin, tetracycline, trimethoprim) or toxic metals/metalloids (sodium arsenite, mercury chloride) as selective agents. This approach yielded 42 strains of which 18 contained plasmids. The selective medium containing trimethoprim allowed the selection of the largest number of isolates and the largest number of plasmids. However, media supplemented with chloramphenicol and kanamycin resulted in the highest percentages of plasmid-containing strains. For the physiological and biochemical characterization of the isolates, the production of hemolysins, anionic biosurfactants and quorum sensing inhibitors was evaluated. Hemolytic activity was detected in isolates belonging to the genus *Bacillus*, *Pseudomonas*, *Psychrobacter*, *Shewanella* and *Vibrio*. None of the isolates showed production of anionic biosurfactants. Isolates of the genera *Pseudomonas*, *Vibrio* and *Bacillus* caused quorum sensing inhibition.

Considering the data obtained from functional screening, in future works, we intend to sequence the plasmids obtained and search for genes encoding for metabolic pathways involved in the degradation of specific molecules, such as N-Acyl homoserine lactones and for other families of bioactive compounds of biotechnological interest.

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List of Abbreviations

ARS- Sodium Arsenite

BLAST-N- Basic Local Alignment Search Tool – Nucleotides

BSF -Biosurfactant

CLOR- Chloramphenicol

CTAB- Cetyl Trimethylammonium Bromide

DGGE- Denaturing gradient gel electrophoresis

FISH- Fluorescence in situ hybridization

HGF- Hepatocyte Growth Factor

HGT- Horizontal Gene Transfer

KAN – Kanamycin

MB- Methylene Blue

MER - Mercuric Chloride

MGE- Mobile Genetic Element

PSC- Porifera Sample Code

qPCR- Quantitative Real-Time Polymerase Chain Reaction

SC- Selective Compound

SDS- Sodium Dodecyl Sulphate

TC-DNA- Total Community DNA

TETRA - Tetracycline

TRACA - Transposon-Aided Capture Method

TRIM- Trimethoprim

TSA - Tryptic Soy Agar

TSB - Tryptic Soy Broth

Introduction

1- Introduction

1.1- Mutualistic relations between bacteria and invertebrates

Bacteria played a very important role on the early stages of life on earth. Fossil evidence shows that the earliest organisms were prokaryotes. This form of life allowed and shaped the origin and evolution of eukaryotes (Alegado & King, 2014). It is likely that these microorganisms acted as food sources and as pathogens and were responsible for several chemical signs that influenced the physiology and morphology of early animal life forms. Beneficial alliances between animals and bacteria shaped evolution of eukaryotic cells allowing them to access otherwise inaccessible ecological niches (Chaston & Goodrich-Blair, 2010; Alegado & King, 2014). Collar cells, for example, are a conserved cellular structure found in sponges, cnidarians and choanoflagellates that suggests that ingestion of bacteria as an energy supply was fundamental to primordial forms of these animals (Mcfall-Ngai *et al.*, 2013; Alegado & King, 2014). Bacterial-host co-evolutionary processes can be explained by two common models, the trench warfare model (or balanced polymorphism model) and the co-evolutionary arms race model (or red queen model). The trench warfare model suggests that diversity in the population is a consequence of selective pressures that preserve polymorphisms (e.g. escape the host immune system). The co-evolutionary arms race model defends that there is back-and-forth evolution amongst targeted eukaryotic molecules and the bacterial proteins (Toft & Andersson, 2010). Many hypotheses on the origin of multicellularity were based on the discovery that *Sapingoeca rosetta*, a choanoflagellate that responds to specific bacterial signals by initiating colony formation through cell division (Mcfall-Ngai *et al.*, 2013).

Symbioses between animals and microorganisms happen very recurrently in every niche. Mutualistic relationships are defined as close and long-term symbiotic interactions with positive effects for both partners (Muller, 2009). These interactions can be facultative or obligate and provide different benefits for the host and the microbe including defense, development, reproduction and nutrition (Chaston & Goodrich-Blair, 2010). The transmission of symbionts can occur by direct transfer from parent to offspring (vertical transmission), by the spread of symbionts amongst neighbour hosts (horizontal transmission) and/or by reinfection of the new host generation from the environmental

bacterial stock (Wahl *et al.,* 2012). The human gut microbiota serves as an excellent example of how microbial-host interactions can strongly influence human health playing a vital role in metabolic regulation and in the immune system (Li *et al.,* 2016). The human gut hosts a rich and diverse microbial community. However, many invertebrates such as aphids, sharpshooters and stinkbugs houses simple communities of microorganisms (Zaneveld *et al.,* 2008). Invertebrate phyla that populate marine environments establish mutualistic interactions with bacteria (Chaston & Goodrich-Blair, 2010; Blunt *et al.,* 2016; Moulton *et al.,* 2016). Invertebrates are interesting models for the understanding of bacteria-host interaction. Some show relatively low variety of symbiotic bacteria, which simplifies the study of the molecular, cellular and physiological interfaces between the host and the microorganisms (Chaston & Goodrich-Blair, 2010). In addition to their tendency to associate with relatively simple microbial communities, invertebrates are also interesting because of their diversity and general experimental manageability (Chaston & Goodrich-Blair, 2010).

Ocean is relatively poor in nutrients, but it contains a vast range of substrate and energy sources that can be used by microbial symbionts and transferred to their animal hosts. The first of such symbiotic associations occurred very probably within invertebrates of the marine environment and since this taxonomic group actually exhibit the greatest phyletic variety, it is very likely that their symbiotic associations are the most diverse in the current biosphere (Mcfall-Ngai & Ruby, 2000). Many marine invertebrates that inhabit deep-sea hydrothermal vents (e.g. *Riftia pachyptila*) contain internal protective structures that house sulfur-oxidizing mutualist bacteria that provide them with fixed carbon in return for access to reduced inorganic compounds and oxygen (Dubilier *et al.,* 2008). The Hawaiian Bobtail squid (*Euprymna scolopes*) light organ is another example of this sort of biological interaction since the bioluminescent *Allivibrio fischeri* is responsible for the light production (Blackall *et al.,* 2015*).*

Marine invertebrates and their symbiotic microflora offer a very rich source of information that can reveal a lot about the diversity of host-bacterial associations as well as about the mechanism that originate and allows the persistence of symbioses (Mcfall-Ngai & Ruby, 2000). The generalized molecular mechanism of marine invertebrate-microbial

associations is the following: once the symbionts arrive the specific point of colonization, the host offers the conditions that allow the microorganisms to recognize this location and to be retained there. This process is very selective and it is thought to be mediated by a highly specific immunological cross-reaction, being very similar to antigen agglutination reactions (Selvin *et al.,* 2010). Symbionts allow animals to benefit from prokaryote pathways of biosynthesis, since prokaryote metabolites can be extremely important as chemical defences discouraging predation. Secondary metabolism of bacterial symbionts is very complex, diverse and plays essential ecological roles. In addition, they may also represent a high biotechnological potential (Haygood *et al.,* 1999; König *et al.,* 2006; Newman & Cragg, 2015).

Bioactive compounds originated from marine organisms are grouped in a wide range of chemical families and exhibit unique properties. Statistics from 1985 to 2008 reveal that the vast majority of new compounds obtained from marine organisms were obtained from invertebrates, 75% of those belonging to phylum Porifera (Hu et al., 2011). Still, a large-scale production of marine invertebrates have not shown industrial viability and more and more potential compounds isolated from these organisms were proved to be produced by their microbial symbionts. These organisms contain genes that can be cloned for the biotechnological production of novel and promising bioactive compounds (Hu *et al.*, 2011).

1.2- Marine sponges

Marine sponges are grouped in phylum Porifera, and correspond to the oldest known metazoans with fossil records dating back more than 580 million years (Hentschel *et al.*, 2006; Hardoim & Costa, 2014). Sponges are aquatic, sedentary filter feeders with the ability to filter thousands of litres of water daily (Hardoim & Costa, 2014). Sponges can be found in freshwater systems (e.g. lakes, streams) and oceans and can even dominate in terms of biomass in the tropical reef fauna (Hentschel *et al.*, 2006). Porifera are subdivided into four classes: *Demospongiae* (demosponges), *Hexactinellida* (glass sponges with syncytial organization), *Calcarea* (calcisponges) and *Homoscleromorpha*. There are at least 8553 species of sponges formally described, 83% belonging to the class of demosponges, a monophyletic lineage characterized by cellular organization that frequently contain a siliceous or organic skeleton (Adamska, 2016; Hardoim & Costa, 2014).

Porifera are the simplest form of multicellular animals since their cells retain some degree of independence and totipotency (Muller, 2009; Perdicaris *et al.*, 2013; Santos-Gandelman *et al.*, 2014). In general they are morphologically diverse since they grow in different sizes (from millimetres to meters), shapes (e.g. rope, encrusting, ball, rube, vase, barrel) and colours (e.g. yellow, white, green, brown, black, blue). These phenotypical characteristics are influenced not only by the genotype of the sponge but also by the environmental conditions (Hentschel *et al.*, 2006). Sponges are mostly hermaphroditic, capable of asexual (e.g. budding) and sexual (vivipary or ovipary) reproduction (Taylor et al., 2007). The basic body plan of a marine sponge contains different cell layers but there are no true tissues, organs or body symmetry (Muller, 2009; Santos-Gandelman *et al.*, 2014). **Figure 1** shows a structural overview of a typical demosponge. Pinacocytes are epithelial cells that compose the outer surface of the animal, also known as pinacoderm. Pinacoderm contains also small inhalant openings called ostia, which extend along the interior channels making the whole structure permeable. Inside a sponge, there are series of chambers containing specialized flagellated cells (choanocytes) that collectively are called the choanoderm (Santos-Gandelman *et al.*, 2014). As filter feeders, sponges transport seawater through the pores (ostia) in the outer pinacoderm tissue due to a flow created by the beating of flagellated choanocyte cells surrounding the aquiferous system. Choanocytes have the function of capturing food particles, which include bacterial cells, viruses and

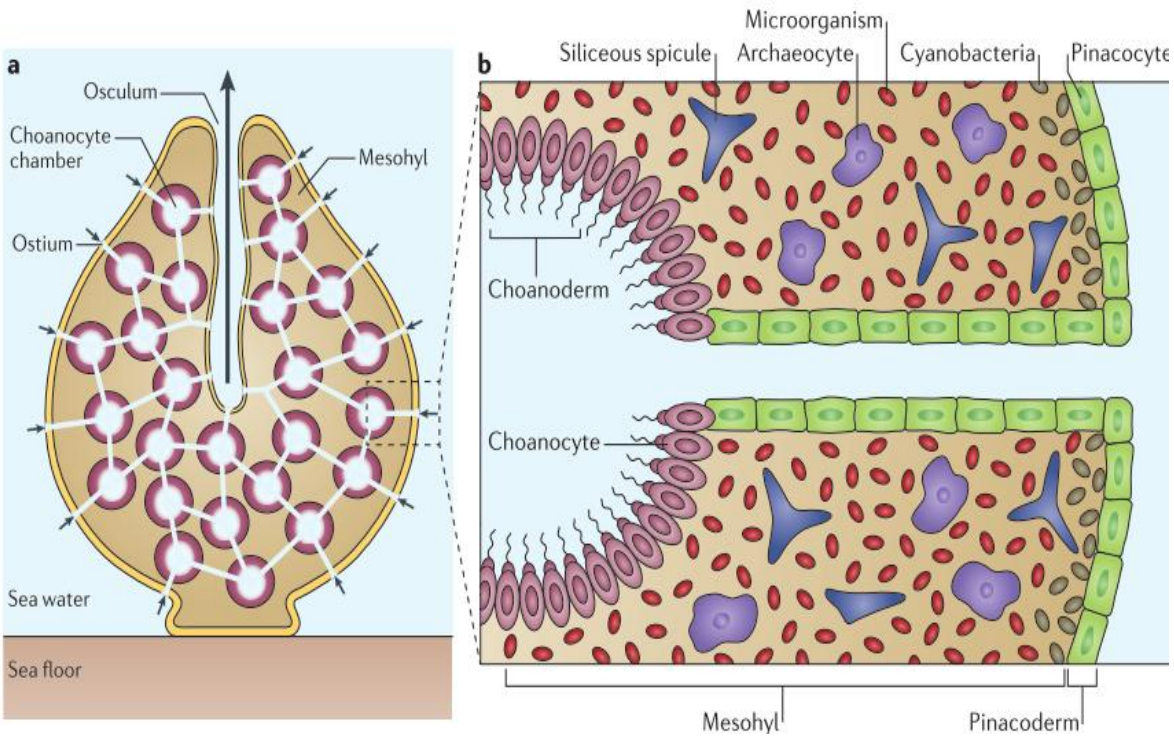

Figure 1*- Schematic representations of the whole structure (**a**) and an enlargement of the internal structure (**b**) of a typical demosponge (Hentschel et al., 2012)*

unicellular algae. However, the sponge nutrition is mainly based on bacteria (Hentschel *et al.,* 2012; Adamska, 2016). After capture, microorganisms are engulfed by archaeocyte cells in the inner mesohyl layer. The seawater is filtrated and rejected through the exhalant opening (also known as osculum); where up to 96% of microorganisms are removed in this process (Hentschel *et al.,* 2012; Santos-Gandelman *et al.,* 2014). Most sponges contain spicules, which provide structural support. Spicules are composed of amorphous, hydrated and noncrystalline silica (SiO_2/H_20) or calcium carbonate ($CaCO_3$) and are produced in specialized cells called sclerocytes. The space between the cell layers surrounding the sponges called mesohyl, which is composed mostly of functionally independent cells (Muller, 2009). Various microorganisms can reside temporarily or permanently in the mesohyl of demosponges (e.g. cyanobacteria, heterotrophic bacteria, unicellular algae, fungi, zoochlorellae). In some species of sponges, up to 60% of their biomass is composed of microorganisms (Selvin *et al.,* 2010; Santos-Gandelman *et al.,* 2014).

1.3- Bacterial symbionts of Sponges and their biotechnological potential

Sponge-microbe associations are common among sponge taxa and some are actually unique to these organisms. These associations are thought to be more truly mutualistic than the temporary associations that result essentially from the activity of the filter-feeding system (Webster & Blackall, 2009; Selvin *et al.,* 2010). Bacteria symbionts constitute up to 60% of the mesohyl, mostly occurring as intracellular symbionts (Webster & Blackall, 2009; Selvin et al., 2009). Sponges' endosymbionts live either inside the sponge cells or in the sponge mesohyl. They can also host epibionts, which live in their surface (Selvin et al., 2009). Mutualistic interactions with bacteria allows several benefits to sponges including supplemental nutrition, production of secondary metabolites (which can act as defense enzymes against pathogens), enhancement of the rigidity of the sponge skeleton and protection from ultraviolet solar radiation (Selvin *et al.,* 2010; Thacker & Freeman, 2012). Bacterial symbionts also benefit by the protection offered by sponges, which happens only if bacteria are not digested by the sponge or if there is a positive balance between growth and mortality rates (Muller, 2009). Different kinds of interactions between sponges and microorganisms are summarized in **Figure 2.**

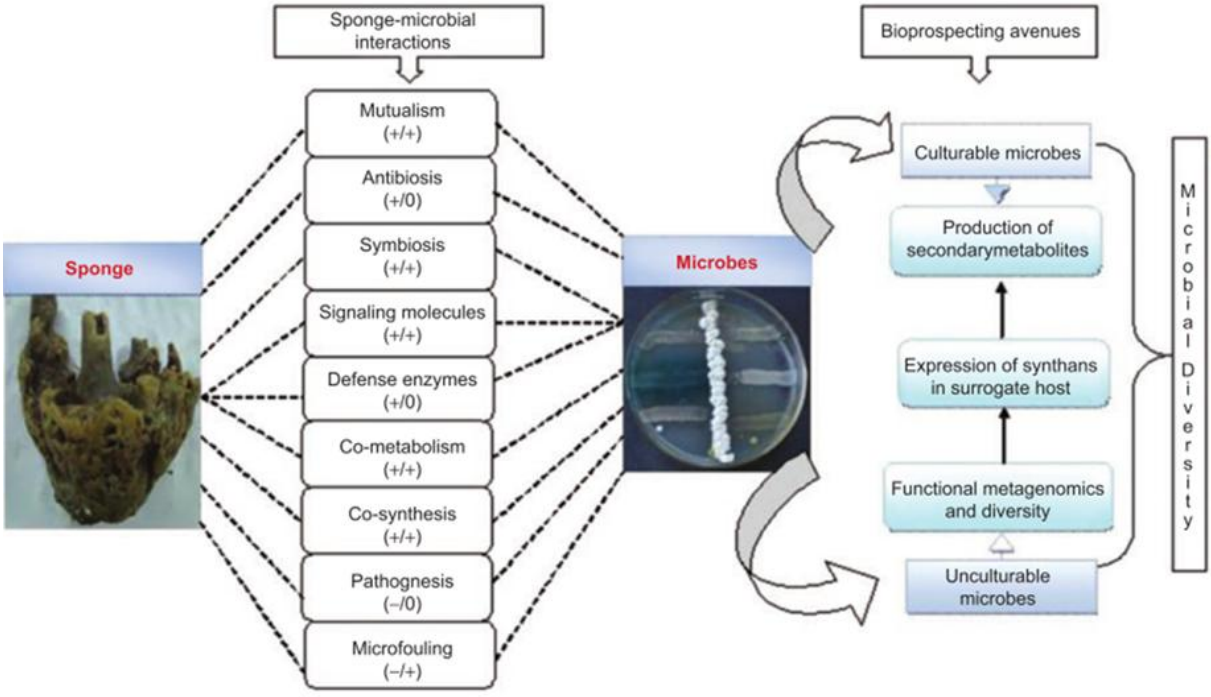

Figure 2- *Hypothetical mechanisms of microbe-sponge interactions and different approaches to explore the microbiome of sponges (Adapted from Selvin et al., 2010).*

Some bacteria can also act as pathogens to specific species of sponges; however, there are not many studies identifying causative agents of diseases in sponges. As an example of a pathogenic bacterium in sponges, there is *Pseudoalteromonas agarivorans* that produces a collagenase enzyme that damages the skeletal fibres of *Rhopaloeides odorabile* (Thacker & Freeman, 2012; Choudhury *et al.*, 2015).

Experiments have suggested that sponge hosts may recognize particular microorganisms through antibiosis. This biological mechanism is based on the capacity of some bacteria to adapt to the presence of antimicrobial molecules produced by the host. When microorganisms reach the target tissue, a shift from an initiation to a persistence approach will involve both the bacterium and the host cells. Bacteria may induce significant physiological changes on sponges through different methods including lipopolysaccharide-induced apoptosis. Once inside the host, microorganisms proliferate due to the favourable conditions found, particularly near choanocyte chambers because of the filtration mechanism (Selvin *et al.,* 2010). As an example of recent research in this matter, the symbiotic *Bacillus* sp. strain from the freshwater sponge *Metania reticulate* produces nitroaromatic compounds that induce specific antibiosis on *Staphylococcus aureus* while inhibiting the degranulation of RBL–$2H_3$ cells, which suggests that the symbiont interacts positively with the host immune system, giving it competitive advantage (Rozas *et al.* 2016).

Although there is a great diversity among symbiotic communities, the major research interest have been set on the bacterial endosymbionts of sponges. The mechanisms of interaction between sponges and endosymbionts is not yet completely well understood. however it is thought to follow similar principles of the generalized molecular mechanisms of marine invertebrate-microbial associations, as it is shown on **Figure 3**.

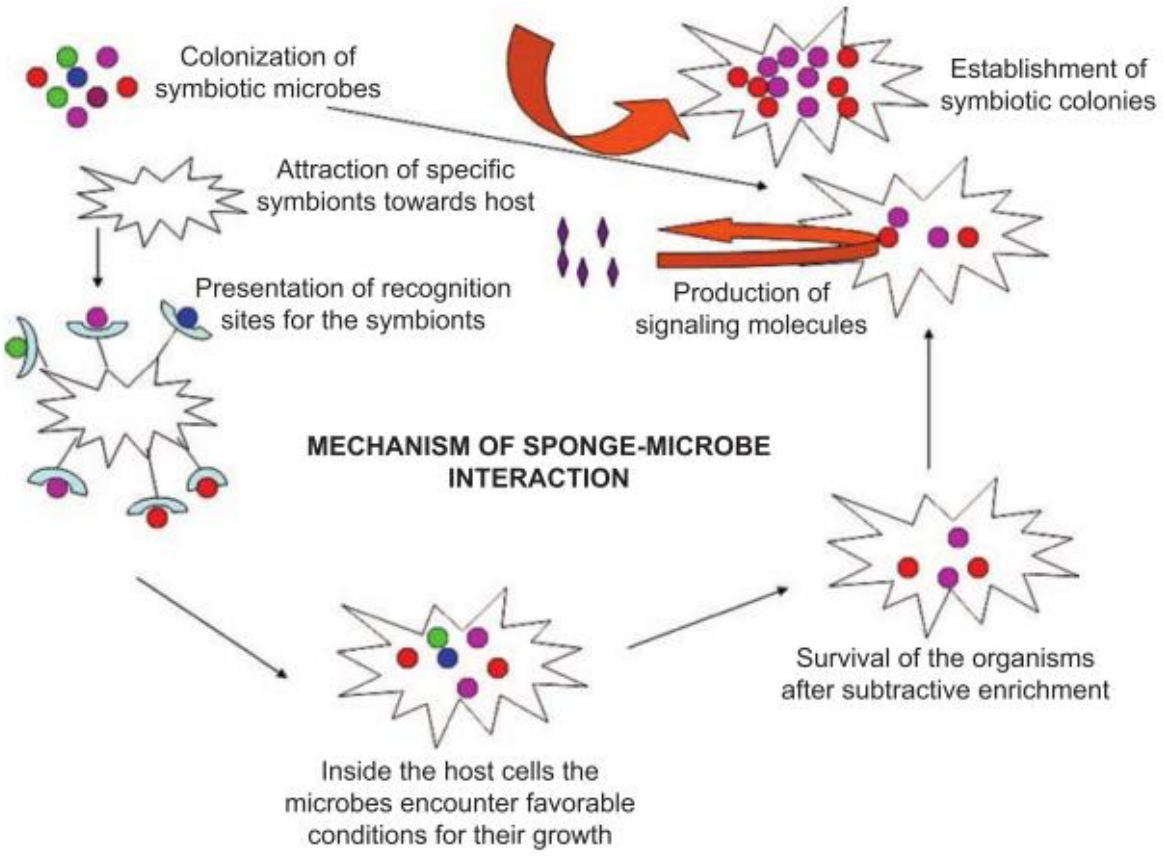

Figure 3- *Hypothetical mechanisms of interaction between sponges and its symbionts (SELVIN et al., 2010).*

The relevance of symbiotic bacteria to Porifera is also highlighted by the existence of mechanisms of bacterial transfer from parents to embryos, which have been demonstrated in several species such as *Spongia* and *Hippospongia* (Muller, 2009). This transfer and restitution supports the hypothesis that a constant specific and possibly symbiotic association with bacteria may be important to the biological success of the sponges (Muller, 2009).

As a whole, sponges and their symbionts are important players in biogeochemical cycles, which can metabolize significant fractions of primary production and return organic carbon to the environment. They mediate complex array of nutrient transformations, releasing labile nutrient forms (e.g. nitrate, nitrite, phosphate, ammonium) from less bioavailable organic molecules (Colman, 2015). Uptake, transformation, and release of carbon and nutrients by the sponge together with its symbiont microbial community may

have a significant impact on the neighbouring water quality and ecosystem function (Colman, 2015).

Recently, advances regarding molecular cultivation-independent techniques, such as 16S rRNA gene analysis, denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH), have been allowing new discoveries regarding the microbiome of marine sponges (Wang *et al.,* 2006). Poribacteria is an example of a new candidate phylum that, despite high abundance and specificity to sponges, was only detected in the last decade, thanks to these progresses (Fieseler *et al.,* 2004). Regardless of the detection technique, studies showed that the most abundant sponge-associated bacterial phyla are Proteobacteria, Chloroflexi, Actinobacteria, Acidobacteria, Nitrospirae and the candidate phylum Poribacteria (Hentschel *et al.,* 2012).

The potential of sponges and their bacterial symbionts as a source of novel bioactive compounds can be inferred from recent scientific literature (Thakur *et al.,* 2004; Santos-Gandelman *et al.,* 2014; Santos *et al.,* 2015). Yet, there is still the ecological perspective these substances and on their role on the distribution and adaptation of sponges to very diverse habitats. Researchers suggest that sponges developed defensive chemical weapons (production of metabolites) through a long evolutionary process, which gave them survival and reproductive advantages (Thakur *et al.,* 2004; Ruzicka & Gleason, 2008; Turon et al., 2009; Blunt *et al.,* 2016). Associations between sponges and bacteria have been intensively studied since it have been showing to be involved in the production of several bioactive metabolites that have a great impact on the fitness of Porifera (Jakowska & Nigrelli, 1960; Muller, 2009; Thacker & Freeman, 2012).

Antibiotic activities found in sponges such as *Microcioma prolifera* motivated researchers to question the ecological role of these biological compounds and the possible role of symbiotic bacteria in their production (Jakowska & Nigrelli, 1960; Muller, 2009). Various sponge-associated bacteria genera, namely *Actinomyces, Aeromonas, Bacillus, Corynebacterium, Flavobacter, Micrococcus, Pseudoalteromonas, Pseudomonas, Streptomyces* and *Vibrio* have been shown to produce antimicrobial molecules (Santos-Gandelman *et al.,* 2014). An example of an antibiotic produced by sponge bacteria is rifamycin, which were only previously known in soil Actinobacteria, and were recently

discovered in a sponge-associated *Actinobacterium* sp. (Kim *et al.,* 2006). Currently, a large number of metabolites has been isolated from marine sponges and associated microorganisms, exhibiting different biological activities namely antibacterial, antitumor, hypocholesterolemic, antimalarial, muscle relaxant, immunosuppressive and anti-inflammatory agents (Perdicaris *et al.,* 2013; Santos-Gandelman *et al.,* 2014; Newman & Cragg, 2015). The most abundant chemical classes of bioactive substances are polyketides, alkaloids, fatty acids, peptides and terpenes (Santos-Gandelman *et al.,* 2014). **Table 1** contains examples of promising substances produced by sponge-associated bacteria.

The main groups of sponge-associated culturable bacteria recognized as probable contributors to pharmacologically relevant substances belong to Firmicutes, Alpha, Beta, Gamma, Delta- Proteobacteria and Actinobacteria. Actinobacteria dominate in the production of therapeutic compounds, followed by Proteobacteria (Santos-Gandelman *et al.,* 2014). The potential of Firmicutes and Cyanobacteria associated to sponges in the production of compounds of biotechnological interest is still unexplored (Santos-Gandelman *et al.,* 2014).

Sponge-associated bacteria are very promising in the context of biomedical research (e.g. new and potent pharmaceutics), industrial biotechnology (e.g. enzymes, biosurfactants) and environmental remediation (e.g. indicators of contamination). Sponges and their microbiome contributed to nearly 30% of all marine natural products so far, which puts Porifera as the richest source of compounds among other marine organisms (Mehbub *et al*., 2014).

Alpha- and Gamma-*Proteobacteria,* which have been isolated from *Mycale laxissima* and *Ircinia strobilina,* produce quorum sensing signalling molecules, such as N-acyl homoserine lactones (AHLs), through which they out-compete other potential settlers and successfully establish within the sponge (Selvin *et al.,* 2010). Quorum sensing regulates processes such as antibiotic production, biofilm formation, cell density and virulence factor secretion (Rutherford *et al*., 2012). The production of quorum sensing inhibition molecules appears as a countermeasure against this kind of intercellular communication (Chen *et al.,* 2013). Quorum quenchers are regarded as promising since they provide an alternative or a complement to antibiotics, which currently is very

important due to the increasing numbers of drug resistant pathogens (Chen *et al.*, 2013; Saurav *et al.*, 2016). Sponge-associated bacteria have also been demonstrated to be a promising source of other high value molecules such as biosurfactants (Dhasayan *et al*. 2014; Santos-Gandelman et al., 2014). Biosurfactants (e.g. rhamnolipids), are currently being industrially applied in pharmaceutic products, cosmetics, detergents and bioremediation protocols (Randhawa & Rahman, 2014). These molecules exhibit functional properties such as dispersion emulsification and foaming (Hu *et al.*, 2015). Due to its unique characteristics, biosurfactants frequently display antibacterial, antibiofilm and hemolytic activities (Santos *et al.*, 2014). Hemolytic activity is often detected in biosurfactants as well a protein compounds. Protein hemolysins are toxins that have the ability to form pores in different biological membranes, which allows several applications in medicine and molecular biology (Choudhury *et al.*, 2015). The highly competitive environment associated to the diverse and dense sponge microbial community may represent a rich source of hemolysins, biosurfactants and quorum quenching molecules.

The exploration of the microbiome of sponges is a promising and challenging field of research (Selvin *et al.*, 2009; Abdelmohsen *et al.*, 2014; Santos-Gandelman *et al.*, 2014; Santos *et al.*, 2015; Indraningrat *et al*., 2016). The process of prospecting for new bacterial bioactive compounds must address the culturable and unculturable fractions of the microbiome of sponges and the combined use of metagenomics and metabolomics approaches is likely trigger a revolution in the field of marine-compound-based drug development, in the short term (Hentschel *et al.*, 2012; Indraningrat *et al*., 2016).

***Table 1**- Antimicrobial substances produced by bacteria associated to marine sponges (Adapted from Santos-Gandelman et al., 2014)*

Substance	Biological Activity	Bacteria	Sponge	Reference
Triclosan and Lutoside	Antibacterial	*Micrococcus luteus* R-1588-10	*Xestospongia* sp.	Bultel-Poncé *et al*., 1998
2-undecyl-4-quinolone	Antimalarial/ Anti-HIV	*Pseudomonas* sp. 1537-E7	*Homophymia* sp.	Bultel-Poncé *et al*., 1999
Clyclo-(glycil-L-seryl-L-prolyl-L-glutamyl)	Antibacterial	*Ruegeria* sp.	*Suberites domuncula*	Mitova *et al.*, 2004
Diketopiperazine	Antibacterial	*Micrococcus* sp.	*Tedania ignis*	Stierle & Cardeliina, 1988
Manzamine A	Antimalarial	*Micromonospora* sp.	*Acanthostrogylophora* sp.	Ang *et al.*, 2000
Pyrone-I	Antibacterial/ Antifungal	*Pseudomonas* sp. F92S91	Unidentified	Sing *et al.*, 2003
Surfactin, Iturin and Fengycin	Antibacterial/ Antifungal	*Bacillus subtilis* A184	*Aplysina aerophoba*	Pabel *et al*., 2003
Tetromycin B	Antiprotozoal	*Streptomyces axinellae* Pol001	*Axinella polypoides*	Pimentel-Elardo *et al.*, 2011
Thiopeptides	Antibacterial	*Bacillus Cereus* QN03323	*Halichondria japonica*	Nagai *et al.*, 2003

1.4- Bacterial plasmids: ecological role and biotechnological importance

Prokaryotes from the *Archaea* and *Bacteria* domains reproduce by asexual cell division, which involves the replication of the genomes and produces two identical cells. In this process, changes are introduced largely by mutations and occasionally by intragenomic duplication of the pre-existing genetic information, which leads to the production of altered proteins (Garcia-Vallvé *et al.* 2000; Narra & Ochman, 2006; Liu & Ochman, 2017). However, genetic changes can also be introduced by horizontal gene transfer (HGT), which plays an important role in bacterial diversity and adaptability (Heuer & Smalla, 2012). Bacteria can acquire resistance vertically (e.g. through mutations). However, resistance development is mostly associated to HGT (Högberg *et al.*, 2010). HGT is induced by the presence of mobile genetic elements (MGEs) such as transposons, plasmids and viral vectors. All MGEs present in a cell compose its mobilome (Siefert, 2009). In contrast to mutations, HGT allows a faster and more efficient adaptation to new habitats and environmental changes (Heuer & Smalla, 2007). HGT is mediated by three main processes: conjugation, transduction and transformation. Conjugation consists in the transfer of DNA by direct contact between cells, which relies regularly on conjugative plasmids, while transduction depends on a phage vector to introduce foreign DNA in the recipient cell. Transformation is the process of uptake of exogenous DNA from the environment by the bacteria. Although all the mentioned mechanisms allow bacterial adaptation, conjugation and transduction depend on semi-autonomous vectors – conjugative elements and temperate phages, respectively (Harrison & Brockhurst, 2012).

Plasmids are extrachromosomal genetic elements (DNA molecules) present in bacteria that carry genes that often empower the cell with advantageous capacities. These genes are arranged in discreet operons, which compose a genetic mosaic from different sources. Plasmid genetic structure is a consequence of frequent recombination. Bacterial plasmids are mainly circular but they can be also linear (Brown, 2011). The structure of plasmids can be divided into a core "backbone" of genes encoding key functions (replication, segregation and conjugation) and "accessory" genes that encodes advantageous traits (e.g. virulence, resistance to antimicrobials, degradation of substrates, metabolic functions) to the bacterial host (Summers, 2002; Harrison & Brockhurst, 2012). Plasmids contain at least one gene that acts as an origin of replication (ori) which allows

them to proliferate independently of the bacterial chromosome. Plasmid sizes vary from approximately 1 kb to over 250 kb. The replication of smaller plasmids depends on the DNA replicative machinery of the cell, while some larger plasmids contain genes that control plasmid replication (Brown, 2011).

Plasmids are commonly classified according to the genes carrying the characteristics conferred to the host bacteria, defining different main types (Brown, 2011). Resistance or R-plasmids are the most common plasmids. They contain genes that confer resistance to antibacterial agents (e.g. Rbk, IncA/C) (Brown, 2011; Tagg *et al.*, 2014; Hennequin & Robin, 2016). Fertility or conjugative plasmids are able to direct conjugation between different bacteria of the same, or related, species (e.g. F-plasmid of *Escherichia coli*). This class of plasmid contains genes that allow cell-to-cell horizontal transfer through a conjugative structure, usually in the form of a pilus (Harrison & Brockhurst, 2012). Killer plasmids carry genes that encode for toxins, used to kill other bacteria. This type of plasmid is not widespread and is general absent from common bacteria. However, as an example, Col plasmids carrying genes that encode for toxic proteins (colicins) occur in toxigenic *E. coli* strains (Brown, 2011; Braun *et al.*, 2015). Degradative plasmids are important in the metabolism of the bacterial host, allowing them to metabolize unusual molecules like salicylic acid and toluene (e.g. TOL plasmid of *Pseudomonas putida*). Virulence plasmids are responsible for the pathogenicity of the host bacterium. The Ti plasmid of *Agrobacterium tumefasciens*, which causes crown gall disease in dicotyledonous plants, is an example of the category (Brown, 2011).

The carriage and replication of plasmids is energetically costly and a physiological burden on the host cell due to the upkeep and repair of plasmid DNA and the production of plasmid proteins involving the host cellular machinery. In addition, the production of conjugative pili exposes the cell to eventual attacks from pilus-specific bacteriophages (Harrison & Brockhurst, 2012). Maintenance of plasmids and their accessory genes in bacterial populations can be explained in a coevolution perspective in which co-adaptation plays a major role. In that context, coevolution consists in changes in fitness (or other traits) that are associated with adaptation in both plasmid and bacteria. Selective environmental pressure (e.g. presence of antibiotics) will determine the balance between costs and benefits

associated with maintaining plasmids, or, in other terms, if keeping the plasmid will be advantageous to the cell (Harrison & Brockhurst, 2012). Co-culture studies show that plasmid co-adaptation broadens plasmid persistence through amelioration mechanisms, which include changes in conjugation rate, changes in plasmid gene expression and loss of plasmid genes (Harrison & Brockhurst, 2012). Beneficial plasmids genes may also be transferred to the bacterial chromosome, which avoids the costs of plasmid carriage (Rankin e*t al.,* 2011). MGEs, including plasmids, may also have crucial roles in the establishment of mutualistic relations between prokaryotes and eukaryotes (Rankin e*t al.*, 2011). Symbiotic plasmids may encode for useful or even essential characteristics in mutualistic relations with animals and plants such as nitrogen fixation (e.g. *Rhizobium* sp., *Burkholderia phymatum*) and production of amino acids (e.g. *Buchnera aphidicola*) (Wenergreen & Moran, 2001; Rankin e*t al.,* 2011; Moulin e*t al.,* 2015*;* Peres-Carrascal *et al.,* 2016).

Attempts to access environmental MGEs involve several methodological approaches for plasmid isolation. Endogenous isolation of plasmids is a commonly used technique that depends directly on the culturable fraction of bacteria in a particular environment. Bacteria are isolated in either non-selective or selective media (e.g. containing antibiotics, heavy metals, xenobiotics). Pure cultures are then screened for the presence of plasmids through extraction of plasmid DNA. Endogenous isolation contains the advantage that the bacterial host can be readily identified. Exogenous isolation of plasmids relies on the transfer of MGEs to an external host, through HGT, which allows obtaining plasmids independently of the culturability of their original hosts (Smalla & Sobecky, 2002). Exogenous plasmid isolation can be done by means of biparental and triparental mating. Exogenous biparental mating consists in mixing environmental bacteria with recipient cells, in which after a filter mating the cells are re-suspended and plated on media supplemented with specific selective substances (e.g. kanamycin (Kan) and rifampicin (Rif)) used for the selection of recipient cells and a substance to which the recipient was originally sensitive (e.g. heavy metals, antibiotics). Triparental mating isolation of plasmids makes use of a second donor carrying a small mobilizable IncQ plasmid so that the capture is entirely based on the mobilizing capacity of the plasmid. Identification of transconjugants can be done through suitable biomarkers such as green

fluorescent protein (gfp) for labelling the recipient cells. Both methods of exogenous isolation can be seen as cultivation-independent. However, a successful transfer requires indigenous cells to have sufficient metabolic activity (e.g. conjugative type IV secretion) since plasmid translocation is an energetically costly process. A disadvantage of exogenous plasmid capturing techniques is that the identity of the original host remains unknown (Smalla *et al.,* 2015).

Cultivation-independent methods represent another approach to plasmid isolation. Total community DNA (TC-DNA) extracted directly from environmental samples can be used for the detection of plasmid occurrence and quantification of abundance. PCR-amplification with primers that target common plasmid sequences of specific groups is required due to low plasmid abundance (Smalla *et al.,* 2015). Quantitative real-time PCR (qPCR) is also an important tool in plasmid ecology since it can elucidate factors that influence plasmid relative abundance in microbial communities of different habitats. Other culture-independent methods, such as the transposon-aided capture method (TRACA) have been successfully used for the isolation of novel plasmids (Dib *et al.,* 2015). This method uses purified plasmid DNA extracted from bacteria, cell cultures or environmental samples which is then amended with an EZ-Tn5 OriV Kan2 transposon and a transposase so that a transposition reaction is promoted *in vitro,* followed by electroporation of the transposition mixture into *E. coli* EPI300 cells. Transformants are then plated in media supplemented with Kan and captured plasmids can be studied. TRACA method has the advantage of capturing plasmids without the requirement of selective markers, but this technique captures preferentially small plasmids (<10 kb) (Smalla *et al.,* 2015).

Plasmids currently play an important role in molecular biology and modern biotechnology, allowing the production of several high-value molecules including antibodies, pharmaceutical proteins and industrials enzymes (Oliveira & Mairhofer, 2013; Prazeres & Monteiro, 2014). Plasmids also have direct application as biopharmaceuticals for therapy or prophylaxis. For instance, plasmids can be useful for the increasing of the expression of specific endogenous proteins by adding additional copies of the coding genes. This approach could be used in the treatment of Parkison's disease since increased expression of the hepatocyte growth factor (HGF) gene may enhance the function of

dopaminergic neurons (Prazeres & Monteiro, 2014). Plasmid biopharmaceutics also can act by replacing single genes containing hereditary defects (e.g. as in Duchenne muscular dystrophy and cystic fibrosis). Regular levels of the normal protein can be produced in the organism by transferring the correct gene via plasmids (Prazeres & Monteiro, 2014).

Marine environments correspond to the largest fraction of the biosphere and still hold an enormous biotechnological potential. Marine mobilome is a decisive driver of fast genome diversification, especially under environmental stress, which reflects on bacterial survival and proliferation. A relatively high fraction (approximately 52%) of the microorganisms found in marine habitats (e.g. water column, sediments, salt marsh rhizospheres) contains one or more plasmids (Sobecky & Hazen, 2009). Isolation and identification of marine MGEs may reveal important and unexpected aspects of gene flux and molecular functions of those elements, as well as open perspectives to the production of novel high-value biomolecules (Sobecky & Hazen, 2009; Smalla *et al.*, 2015).

Sponges are no exception regarding the richness of the bacterial mobilome. The existence of large microbial populations within the mesohyl of sponges, the filtration of large volumes of seawater and the accumulation of contaminants (e.g. heavy metals) in sponges are main reasons for the dissemination of antibiotic and heavy metal resistance through plasmids, in sponge-associated bacteria (Selvin *et al.*, 2009). As an element of the sponge filter-feeding system, the mesohyl works as a natural collector or accumulator of bacteria (Muller, 2009) and represents a competitive microhabitat in which bacteria carrying plasmids are expected to have an advantage. The transference of mobile genetic elements transfer is more frequent in environments where bacterial cells are in close proximity to each other and in relatively high densities (Van Hoek *et al.*, 2011), which is the case of the mesohyl of marine sponges (Selvin *et al.*, 2009).

Several studies have reported novel plasmids isolated from bacteria associated to marine sponges. Barbosa *et al.* (2014) identified a novel 5.8 kb erythromycin resistance plasmid from *Bacillus* sp. HS24 isolated from *Haliclona simulans*. The isolated plasmid, pBHS24B had a mosaic structure that carried the erythromycin resistance gene erm(T) – the first report of this determinant in the genus *Bacillus*. Selvin *et al.* (2009) isolated bacteria that showed resistance against several antibiotics and heavy metals (e.g. Hg, Cd)

from *Fasciospongia cavernosa*. These bacteria contained plasmids in different numbers (1-3) and sizes (10.2-20.6 kb) that according to results might carry the resistance factor. Phelan et al. (2011) provided the first report of a tetracycline resistance-encoding plasmid from sponge-associated bacteria. The plasmid pBHS24 was isolated from *Bacillus* sp. associated with the sponge *Haliclona simulans.* Also from this sponge, Selvin *et al.* (2012) isolated a novel halotolerant lipase through functional screening of a fosmid metagenome library. Lipases have several industrial applications (e.g. biocatalysts, baking, detergents) and the activity of this particular enzyme in high salinity, pH and temperature conditions makes it suitable and promising for industrial applications. Isolation and identification of novel environmental plasmids is a very relevant area of research due to importance of MGEs in ecology as well as its biotechnological potential (Smalla *et al.*, 2015).

1.5- Objectives

This study aims at exploring the biotechnological potential of the microbiota of marine sponges through the isolation of plasmids from culturable sponge-associated bacteria and screening of obtained isolates for the production of new enzymes and/or new biological active compounds.

For this purpose, specific objectives in this work include the endogenous isolation of plasmids using different selective compounds, characterization of bacterial hosts (16S rRNA gene analysis) and detection of biosurfactants, quorum quenching inhibition molecules and hemolysins in the obtained bacterial strains.

Materials and methods

2- Materials and Methods

2.1– Sampling

Marine sponges were collected in Penghu archipelago (western coast of Taiwan) using SCUBA in July and August, 2016. Samples were collected in the following locations: Dongji island (23°15'27.17"N, 119°40'45.28"E), Siji island (23°14'35.80"N, 119°36'53.56"E), Xiyu island (23°34'21.58"N, 119°29'35.14"E), Maoyu islet (23°19'27.62"N, 119°19'20.37"E) and Aimen beach, Magong city (23°33'2.54"N, 119°38'26.83"E). The sponges species obtained were: *Agelas cavernosa, Cinachyrella* sp., *Paratetilla* sp., *Ptilocaulis* sp., *Stylissa carteri*, *Suberites* sp. and *Xestospongia testudinaria.* Samples were identified as PSC followed by a three-digit number. Additional information is described in **Table S1** (Supporting Information). After sampling, the sponge was preserved in marine water with 2% agar, and stored at 4 ºC until processed. In the laboratory (Aveiro, Portugal), the agar was removed and the sponge tissue was inserted into sterile tubes containing Tryptic Soy Broth (TSB) shaking overnight at room temperature. Finally, glycerol (30%) was added to the medium and the samples were stored at -80ºC.

2.2– Isolation of sponge-associated bacteria

Before the isolation process, a pre-enrichment step was carried out using TSB supplemented with the antifungal cycloheximide (100 µg/mL). As a strategy to enrich bacterial populations carrying plasmids containing genes of resistance, different selective compounds (SC) were also added to the medium: kanamycin (KAN) at 30.0 µg/mL, chloramphenicol (CLOR) at 50.0 µg/mL, trimethoprim (TRIM) at 0.5 µg/mL, tetracycline (TETRA) at 5.0 µg/mL, mercuric chloride (MER) at 20.0 µg/mL and sodium arsenite (ARS) at 11.7 µg/mL. Each sponge sample (32 in total) was added to each SC and incubated during 72 hours, at 25ºC, 170 rpm. Abiotic controls of TSB and each SC were also included. Aliquots from the enriched cultures showing bacterial growth (increase of turbidity) were obtained after 24, 48 and 72h. These aliquots were serial diluted (up to 10^{-8}) in NaCl 0.8% and spread-plated in Tryptic Soy Agar (TSA) supplemented with the respective SC. Selective cultures with no visible growth were directly plated on TSA containing its respective SC. The incubation in TSA was performed at 25ºC up to 5 days.

Selected colonies were purified in TSA medium with SC. Pure cultures were grown in TSB supplemented with SC, preserved with glycerol (30%) and stored at -80ºC.

2.3– DNA extraction and genotyping

DNA was extracted from the overnight cultures of the isolated bacteria (TSB with SC) using the method described in Gomes *et al.* (2004) with modifications. Resulting pellets (13000 rpm, 5 min) were resuspended in 800 µL of ethanol and the obtained solution was transferred to 2 mL micro-centrifuge tubes containing 0.5 g of glass-beads (0.10–0.11mm). Samples were homogenized twice by using the FastPrep FP120 bead beating system (Bio 101, Vista, Calif.) at 5.5 m/s for 30 sec. Tubes were centrifuged for 5 min at 16,000 x g and the supernatant was discarded. 1 ml of extraction buffer pH 7.0 (1% hexadecyltrimethyl-ammoniumbromide-CTAB, 2% SDS, 1.5 M NaCl, 100 mM sodium phosphate buffer pH 7.0, 10 mM Tris-HCl pH 7.0 and 1 mM EDTA pH 8.0) was added, samples were mixed by inversion and incubated for 15 min at 65 ºC. Tubes were centrifuged (16,000 x g, 5 min) and the supernatants were transferred to 2 ml tubes containing 1 ml aliquots of phenol-chloroform-isoamyl alcohol (25:24:1) which were centrifuged at 16,000 x g, for 5 min. The upper phases were transferred to new micro-centrifuge tubes, and the nucleic acids were precipitated by addition of 0.6 volume of isopropanol, incubation for 30 min at room temperature and centrifugation (16,000 x g, 20 min). Supernatants were discarded, pellets containing DNA were dried (55 ºC, 10 min), resuspended in 80 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and finally stored at -20ºC.

Bacterial strains were grouped by the comparison of the genomic fingerprints obtained with BOX-PCR (**Table 2**). Reaction mixtures (25 µL) contained 8.75 µL MilliQ water, 1.25 µL of Dimethyl sulfoxide (DMSO), 1.5 µL of BOX-A1R primer (0.6 µM), 12.5 µL DreamTaq^{TM} PCR Master Mix (Fisher Scientific), and 1 µL of template DNA diluted 1/10. The amplification conditions were as follows: initial denaturation (94 °C for 7 min), 35 cycles of denaturation (94 °C for 1 min), annealing (53 °C for 2 min) and extension (65 °C for 8 min), and final extension (65 °C for 16 min). 6 µL of PCR products were analyzed by electrophoresis on a 1.5 % agarose gel at 80 V, 120 min. All procedures involving electrophoresis in the present work used Gene Ruler 1kb Plus DNA ladder for DNA sizing.

Gel images were captured using the Bio-Rad's ChemiDoc XRS+ system. Obtained genomic profiles and band positions were analyzed using GelCompar II software and grouped by Bray-Curtis similarity (Single-Linkage) using Primer 7 software.

Table 2 - *Amplification conditions and primers used in the PCR-related techniques*

Primer	Sequence (5' to 3')	Annealing Temperature (ºC)	Primer (µM)	Fragment size (bp)	Reference
1378 R	CGG TGT GTA CAA GGC CCG GGA ACG	56	10	c.473	Heuer *et al.* (1997)
U27F	AGA GTT TGA TCC TGG CTC AG	56	10	c.1450	Weisburg et al. (1991)
BOX-A1R	CTA CGG CAA GGC GAC GCT GAC G	53	10		Martin *et al.* (1992)

2.4– Detection of plasmids

Strains were grown in TSB supplemented with the respective SC and were centrifuged at 4400 rpm for 15 min. Pellets were resuspended in $Mg.SO_4.7H_20$ (10 mM) and then centrifuged again (13000 rpm, 5 min). The resulting pellets were used for the plasmid extraction method, using the QiAprep Spin Miniprep kit according to the manufacturer instructions. Finally, 5 µL of obtained products were analyzed by electrophoresis on a 0.8% agarose gel, at 80 V 30 min. Gel images were captured using the Bio-Rad ChemiDoc XRS+ system.

2.5– 16S rRNA sequencing

Bacterial strains with different genotypes (BOX-PCR genetic fingerprints) or that contained plasmids were submitted to 16S rRNA gene sequencing. 16S rRNA gene was amplified using the primers 1378 R and U27F (**Table 2**). Reaction mixtures (25 µL each) contained 10 µL MilliQ water, 1 µL of 2 mg/mL bovine serum albumin (BSA), 0.25 µM of each primer, 12.5 µL DreamTaq^{TM} PCR Master Mix (Fisher Scientific), and 1 µL of template DNA (diluted 1/10). The conditions of amplification were the following: initial denaturation (94 °C for 5 min), 35 cycles of denaturation (94 °C for 45 s), annealing (56 °C for 45 s) and extension (72 °C for 1.5 min), and a final extension (72 °C for 10 min). 5 µL

of PCR products were analyzed by electrophoresis on a 1% agarose 80 V, 30 min. Gel images were captured using the Bio-Rad's ChemiDoc XRS+ system. Obtained products were submitted to Sanger sequencing (GATC, Konstanz, Germany). Partial 16S rRNA gene sequences were compared with different sequences available in the GenBank database using BLAST-N (Basic Local Alignment Search Tool - Nucleotides).

2.6– Functional screening of isolated bacteria

Bacterial isolates selected as representative clones as well as strains containing plasmids were characterized to functional/physiological traits by a set of culture-dependent tests.

2.6.1- Production of hemolysins

Grown cultures of bacteria (TSB, 25ºC, 170 rpm) were streaked in Sheep Blood Agar (5 %, Liofilchem), incubated for 5 days at 25ºC and checked for hemolytic activity (Carrillo *et al*, 1996). Two independent assays were performed.

2.6.2- Production of anionic biosurfactants

A method for evaluating the production of anionic biosurfactants (BSFs) was applied as described in Pinzon & Ju (2009). Grown cultures of bacteria (TSB, 25ºC, 170 rpm) were inoculated in Methylene Blue (MB) Medium (0.2 g/L Cetyl Trimethylammonium Bromide (CTAB), 0.005 g/L Methylene blue, 15 g/L Agar, 1.38 g/L Triptone, 4 g/L $MgSO_4.7H_20$, 1 g/L $Na_2HPO_4.H_20$, 0.005 g/L $CaCl_2.2H_2O$, 25 mL/L Glycerol) and incubated for 48h at 25ºC. Plates were then stored in the refrigerator (4ºC) during 7 days and then observed for the presence of dark blue zones (positive result). *Escherichia coli* Dh5-α was used as negative control and sodium dodecyl sulphate (SDS, 5%) as positive control. Two independent assays were conducted involving 3 replicate streaks per isolate.

2.6.3– Production of quorum-quenchers

The production of quorum sensing inhibition molecules (quorum-quenchers) was evaluated using an adaptation of the method described in McLean *et al.* (2004). This approach relies on the fact that the production of violacein, the purple pigment of the Gram negative bacterium *Chromobacterium violaceum*, is quorum sensing dependent. Depigment

halos on *C. violaceum* growth indicate that the test strain releases a metabolite that blocks chemical communication between *C. violaceum* cells, which inhibits the production of violacein (McLean *et al.*, 2004).

Tested bacteria (TSB supplemented with the respective SCs, overnight, 170 rpm) were inoculated in TSA with a sterile toothpick and incubated for 24h, at 25ºC. A layer of 5 mL full strength Luria Bertani Broth (LB) with 5% (w/v) agar containing 50 µL of a fresh culture of *C. violaceum* (LB, overnight, 25ºC, 170 rpm) was added to the surface of the inoculated TSA plates. Incubation was conducted at 25ºC for 48h. Cinnamaldehyde was used as a positive control and *C. violaceum* as negative control. The presence of depigmented halos (positive result) was checked. Two independent assays were conducted with duplicate streaks per isolate.

Results

3- Results

3.1– Isolation of sponge associated bacteria

In total fifty culturable bacterial isolates were obtained from sponge tissue with different selective cultures (16 in ARS, 8 in CLO, 3 in KAN, 4 in MER, 2 in TETRA and 17 in TRIM). According with the BOX-PCR analysis, these isolates were grouped in 42 representatives (12 in ARS, 6 in CLO, 3 in KAN, 4 in MER, 2 in TETRA and 15 in TRIM) (Figures S1, S2, S3 and S4). BOX-PCR was performed in order to select the clonal representatives for further analysis. Obtained electrophoresis gels and the obtained processed data are displayed in Figures S1, S2, S3 and S4 (Supporting Information).

From BOX-PCR analysis, 42 strains isolated from different species of marine sponges were chosen as clone representatives (12 in ARS, 6 in CLO, 3 in KAN, 4 in MER, 2 in TETRA and 15 in TRIM) and consequently used in further steps. Partial 16S rRNA gene sequences of the selected bacteria were compared to data deposited on GenBank using BLAST-N. These results are summarized in **Table 3**. Sequenced strains, mostly gram-negative bacteria (73.8%), were distributed in three different phyla: Proteobacteria (73.8%), Firmicutes (23.8%) and Actinobacteria (2.4%). Proteobacteria was the most common phylum, and all strains were assigned to class Gamma-Proteobacteria. *Pseudomonas* was the most abundant genus (17 isolates, 40.5%), followed by *Vibrio* (10 isolates, 23.8%) and *Bacillus* (6 isolates, 14.3%). *Shewanella* (3 isolates, 7.1%), *Exiguobacterium* (3 isolates, 7.1%), *Brachybacterium* (1 isolate, 2.4%), *Paenibacillus* (1 isolate, 2.4%) and *Psychrobacter* (1 isolate, 2.4%) were also detected.

Table 3- *Bacterial isolates obtained from Porifera using different selective agents: kanamycin (30.0µg/mL), chloramphenicol (50.0 µg/mL), trimethoprim (0.5 µg/mL), tetracycline (5.0 µg/mL), mercuric chloride (20.0 µg/mL) and sodium arsenite (11.7 µg/mL). Bacterial isolates were identified with the code DD/PSC/SC (e.g. 24/565/ARS), in which DD represents the time of growth in TSB (e.g. 24 h); PSC reflects the code of the sample (e.g. PSC 565) and SC the selective compound agent in the medium (e.g. ARS).*

Sample	Sponge Species	16s rRNA Identity	% Similarity	Accession number*
24-1/526/ARS	*Agelas cavernosa*	*Shewanella* sp.	99%	KJ748462.1
72/526/ARS	*Agelas cavernosa*	*Bacillus cereus* group	99%	KY027138.1
48/526/CLOR	*Agelas cavernosa*	*Pseudomonas* sp.	99%	KX817279
48/526/KAN	*Agelas cavernosa*	*Vibrio* sp.	99%	KU321337.1
24/526/TRIM	*Agelas cavernosa*	*Pseudomonas* sp.	100%	KY606932.1
24/394/ARS	*Cinachyrella* sp.	*Exiguobacterium* sp.	100%	KX233849.1
24-1/394/ARS	*Cinachyrella* sp.	*Bacillus* sp.	100%	KY773580.1
24/394/TRIM	*Cinachyrella* sp.	*Bacillus cereus*	100%	KY773581.1
72-2/394/CLOR	*Cinachyrella* sp.	*Pseudomonas* sp.	99%	KT731539
48/394/MER	*Cinachyrella* sp.	*Exiguobacterium* sp.	100%	KY672918
48/395MER	*Cinachyrella* sp.	*Exiguobacterium* sp.	99%	KY672918
72-1/397/TETRA	*Cinachyrella* sp.	*Brachybacterium* sp.	100%	KU560422.1
24/575/TRIM	*Cinachyrella* sp.	*Bacillus cereus* group	100%	KY454692.1
24/576/TRIM	*Cinachyrella* sp.	*Bacillus cereus* group	100%	KY773581.1
24/571/TETRA	*Cinachyrella* sp. *or Paratetilla* sp.	*Vibrio* sp.	100%	KP789964.1
24/572/ARS	*Cinachyrella* sp. *or Paratetilla* sp.	*Vibrio* sp.	99%	CP019118.1
48/572/ARS	*Cinachyrella* sp. *or Paratetilla* sp.	*Pseudomonas* sp.	99%	KX817279.1
48/572/TRIM	*Cinachyrella* sp. *or Paratetilla* sp.	*Pseudomonas* sp.	99%	KY606932.1

Table 3 (Continued)

Sample	Sponge Species	16s rRNA Identity	% Similarity	Accession number*
24-1/446/TRIM	*Ptilocaulis* sp.	*Shewanella* sp.	99%	KU534582.1
24-2/446/TRIM	*Ptilocaulis* sp.	*Vibrio* sp.	100%	KT986142.1
72/446/TRIM	*Ptilocaulis* sp.	*Pseudomonas* sp.	99%	KY606932.1
72/448/CLOR	*Ptilocaulis* sp.	*Pseudomonas* sp.	100%	KX817279
24/449/ARS	*Ptilocaulis* sp.	*Vibrio* sp.	100%	FM957481.1
24/449/TRIM	*Ptilocaulis* sp.	*Vibrio* sp.	99%	CP009978.1
48/449/MER	*Ptilocaulis* sp.	*Shewanella* sp.	100%	KJ710697
48/522/ARS	*Suberites* sp.	*Bacillus* sp.	100%	KY773609.1
48/522/KAN	*Suberites* sp.	*Paenibacillus* sp.	99%	HG964474
24/523/ARS	*Suberites* sp.	*Pseudomonas* sp.	99%	KX817279.1
48/523/CLOR	*Suberites* sp.	*Pseudomonas* sp.	99%	KY606932.1
24/523/TRIM	*Suberites* sp.	*Pseudomonas* sp.	99%	KY606932.1
24-1/524/ARS	*Suberites* sp.	*Vibrio* sp.	99%	KU891054.1
24/524/TRIM	*Suberites* sp.	*Vibrio* sp.	100%	KU534536.1
24/525/CLOR	*Suberites* sp.	*Pseudomonas* sp.	100%	KY606932.1
48/525/TRIM	*Suberites* sp.	*Pseudomonas aeruginosa*	99%	KY769875.1
72-1/410/ARS	*Xestospongia testudinaria*	*Psychrobacter* sp.	99%	JF711009
24/486/KAN	*Xestospongia testudinaria*	*Vibrio* sp.	99%	KJ093445.1
48/488/MER	*Xestospongia testudinaria*	*Pseudomonas stutzeri*	100%	KT986148
24/492/TRIM	*Xestospongia testudinaria*	*Vibrio* sp.	99%	KU534583.1
24/565/ARS	*Xestospongia testudinaria*	*Pseudomonas* sp.	99%	KX817279.1

Table 3 (Continued)

Sample	Sponge Species	16s rRNA Identity	% Similarity	Accession number*
48/565/CLOR	*Xestospongia testudinaria*	*Pseudomonas* sp.	99%	KY606932.1
24/565/TRIM	*Xestospongia testudinaria*	*Pseudomonas* sp.	100%	KX349990.1
48/568/TRIM	*Xestospongia testudinaria*	*Pseudomonas* sp.	100%	KY606932.1

*GenBank sequence Accession numbers of the most related bacterial sequence.

3.2– Detection of plasmids

Agarose gel electrophoresis allowed the detection plasmid DNA in 18 of 42 (42.9%) bacterial isolates. Plasmid positive strains belong to genera *Pseudomonas* (13 strains, 72.2%), *Exiguobacterium* (2 strains, 11.1%), *Vibrio* (2 strains, 11.1%) and *Paenibacillus* (1 strain, 5.6%). Plasmids were classified according to their size (uncut plasmid), which was estimated by comparing the position of their bands in agarose gel electrophoresis with Gene Ruler 1kb Plus DNA ladder. Band positions in the agarose gel indicated sizes between 2 and 20 kbp. However, this does not indicate its real size since uncut plasmid DNA can appear in several forms (e.g. nicked, circular, linear covalently closed, supercoiled, circular single-stranded), which influences strongly the migration and final position in the gel (Schvartzman *et al.*, 2010; Lee *et al.*, 2012; Sinden, 2012). The size of DNA influences the band position; however, it is also affected by the percentage of agarose, time of electrophoresis and the degree of supercoiling (Schvartzman *et al.*, 2010; Lee *et al.*, 2012).

TRIM was the SC that allowed obtaining the highest number of strains containing plasmids (7 strains, 46.7% of TRIM isolates). However the highest percentages of plasmid-carrying strains were produced by CLOR (4 strains, 66.7% of CLOR isolates) and KAN (2 strains, 66.7% of KAN isolates). Furthermore, one strain (48/394/MER, *Exiguobacterium* sp.) had two different bands, while others had just one visible band. These results are summarized in **Table 4**.

Table 4- *Bacterial strains showing evidence for the presence of plasmid DNA in agarose gel electrophoresis*

Strain	Size (kbp)	16s rRNA gene identity
24/523/ARS	2-5	*Pseudomonas* sp.
24/565/ARS	2-5	*Pseudomonas* sp.
48/572/ARS	2-5	*Vibrio* sp.
48/523/CLOR	2-4	*Pseudomonas* sp.
24/525/CLOR	2-4	*Pseudomonas* sp.
48/526/CLOR	2-4	*Pseudomonas* sp.
48/565/CLOR	2-4	*Pseudomonas* sp.
48/522/KAN	2-3	*Paenibacillus* sp.
48/526/KAN	4-5	*Vibrio* sp.
48/394/MER	5 and 20	*Exiguobacterium* sp.
48/395/MER	20	*Exiguobacterium* sp.
72/446/TRIM	3-5	*Pseudomonas* sp.
24/523/TRIM	3-5	*Pseudomonas* sp.
48/525/TRIM	3-5	*Pseudomonas aeruginosa*
24/526/TRIM	3-5	*Pseudomonas* sp.
24/565/TRIM	3-5	*Pseudomonas* sp.
48/568/TRIM	3-5	*Pseudomonas* sp.
48/572/TRIM	2-5	*Pseudomonas* sp.

3.3– Functional/biochemical screening of isolates

The results on the blood hemolysis test were interpreted as α, β or γ hemolysis (**Table 5**). Partial or α hemolysis is reported by the presence of a dark greenish halo. Total or β hemolysis is associated to the presence of transparent halos. Lack of hemolysis (γ hemolysis) corresponds to bacterial growth with the absence of a hemolytic halo (Seeman

et al., 2009). α hemolysis was observed for 21.4 % of all analysed isolates, while 16.7 % and 61.9 % revealed either β or γ hemolysis, respectively. Partial hemolysis was only observed in *Vibrio*, *Psychrobacter* and *Shewanella* isolates and none of them carried plasmids. Additionally, 50 % of isolated *Vibrionaceae* were α hemolytic (5 strains), while the other half did not cause hemolysis. All *Shewanella* (3 strains) and *Psychrobacter* (1 strain) produced a partial hemolysis result. β hemolysis was observed in strains of *Bacillus* (6 strains) and *Pseudomonas* (1 strain). Moreover, 100 % *Bacillus* sp. isolates showed β hemolysis. The *Pseudomonas* 48/525/TRIM was the only *Pseudomonas* strain that showed total hemolysis (β). All the other strains, affiliated with *Brachybacterium*, *Exiguobacterium*, *Vibrio*, *Pseudomonas* and *Paenibacillus* showed γ hemolysis.

None of bacteria showed evidence of production of anionic BSFs as detected by the CTAB-methylene blue agar assay (**Table 5**), since there was no formation of blue halos around the grown bacterial colonies. Negative control (*E. coli* DH5-α) did not produce halos, while positive controls (SDS solution) caused the formation of the characteristic blue circular areas.

The results of the tests for production of quorum sensing inhibition molecules are summarized in **Table 5**. The result was interpreted as positive (+) when depigmented halos were detectable, as a strong positive (++) when the halo measured more than 3 mm on average, or negative (-) where there was absence of depigmented zones around the colony. Positive or strong positive results were obtained in 38.1% of tested isolates, being 93.8 % of those Gram-negative bacteria. In addition, 75.0 % of these isolates belonged to *Pseudomonas*, 18.8 % to *Vibrio* and 6.3 % to *Bacillus*. As expected, negative controls (*C. violaceum*) did not cause depigmentation of the test culture, while positive controls (cinnamaldehyde) did.

Table 5- *Results of the tests of haemolysis, inhibition of quorum sensing and production of anionic biosurfactants.*

Sample	16s rRNA identity	Type of Hemolysis	Anionic BSFs	Quorum Quenching	Plasmid Detection
24/394/ARS	*Exiguobacterium* sp.	γ	-	-	-
24-1/394/ARS	*Bacillus* sp.	β	-	-	-
72-1/410/ARS	*Psychrobacter* sp.	α	-	-	-
24/449/ARS	*Vibrio* sp.	γ	-	+	-
48-1/522/ARS	*Bacillus* sp.	β	-	-	-
24/523/ARS	*Pseudomonas* sp.	γ	-	+	+
24-1/524/ARS	*Vibrio* sp.	γ	-	+	-
24-1/526/ARS	*Shewanella* sp.	α	-	-	-
72/526/ARS	*Bacillus cereus group*	β	-	-	-
24/565/ARS	*Pseudomonas* sp.	γ	-	+	+
24/572/ARS	*Vibrio* sp.	α	-	-	-
48/572/ARS	*Pseudomonas* sp.	γ	-	+	+
72-2/394/CLOR	*Pseudomonas* sp.	γ	-	-	-
72/448/CLOR	*Pseudomonas* sp.	γ	-	+	-
48/523/CLOR	*Pseudomonas* sp.	γ	-	+	+
24/525/CLOR	*Pseudomonas* sp.	γ	-	+	+
48/526/CLOR	*Pseudomonas* sp.	γ	-	+	+
48/565/CLOR	*Pseudomonas* sp.	γ	-	-	+
24/486/KAN	*Vibrio* sp.	γ	-	-	-
48/522/KAN	*Paenibacillus* sp.	γ	-	-	+
48/526/KAN	*Vibrio* sp.	α	-	-	+
48/394/MER	*Exiguobacterium* sp.	γ	-	-	+
48/395/MER	*Exiguobacterium* sp.	γ	-	-	+
48/449/MER	*Shewanella* sp.	α	-	-	-
48/488/MER	*Pseudomonas stutzeri*	γ	-	-	-
72-1/397/TETRA	*Brachybacterium* sp.	γ	-	-	-
24/571/TETRA	*Vibrio* sp.	α	-	-	-
24/394/TRIM	*Bacillus cereus*	β	-	++	-
24-1/446/TRIM	*Shewanella* sp.	α	-	-	-
24-2/446/TRIM	*Vibrio* sp.	γ	-	++	-

Table 5 (Continued)

Sample	16s rRNA identity	Type of Hemolysis	Anionic BSFs	Quorum Quenching	Plasmid Detection
72/446/TRIM	*Pseudomonas* sp.	γ	-	-	+
24/449/TRIM	*Vibrio* sp.	α	-	-	-
24/492/TRIM	*Vibrio* sp.	α	-	-	-
24/523/TRIM	*Pseudomonas* sp.	γ	-	+	+
24/524/TRIM	*Vibrio* sp.	γ	-	-	-
48/525/TRIM	*Pseudomonas aeruginosa*	β	-	++	+
24/526/TRIM	*Pseudomonas* sp.	γ	-	-	+
24/565/TRIM	*Pseudomonas* sp.	γ	-	+	+
48/568/TRIM	*Pseudomonas* sp.	γ	-	+	+
48/572/TRIM	*Pseudomonas* sp.	γ	-	+	+
24/575/TRIM	*Bacillus cereus group*	β	-	-	-
24/576/TRIM	*Bacillus Cereus group*	β	-	-	-

Discussion

4- Discussion

The importance of the microbiome of marine sponges, which is diverse and of enormous biotechnological potential, is documented in recent scientific literature (Selvin *et al.,* 2009; Versluis *et al.,* 2016). Several studies have been attempting to assess the potential of Porifera and their microbiota for the production of novel metabolites (Thakur *et al.,* 2004; Santos-Gandelman *et al.,* 2014; Santos *et al.,* 2015). Yet, work focusing mainly on culturable bacteria and on the biotechnological potential and ecological role of plasmids within the bacterial communities of sponge hosts is strikingly scarce. In the current work, bacteria associated with marine sponges were isolated, and tested for the presence of plasmids. For the isolation of bacteria, a rich medium supplemented with different selective agents, including four different antibiotics (CLOR, KAN, TETRA and TRIM) and two metal/metalloids (MERC and ARS) was used. In this way, isolates containing chromosomal and/or extrachromosomal genes coding for resistance to the selective agents (Baker-Austin *et al.,* 2006), would be positively selected. Considering that resistance genes are often associated with mobile genetic elements, this approach was used to increase the probability of isolating plasmid-carrying strains (Brown, 2011; Gullberg *et al.,* 2014). In addition, due to the possibility of selecting desired characteristics, the use of selective media is often an important prerequisite for the isolation of novel and/or potentially interesting bacteria for biotechnological applications (Sharma *et al.,* 2011; Jana *et al.,* 2014).

The TRIM selective culture produced the highest number of isolates. In agreement with this finding, Santos-Gandelman *et al.* (2013) isolated bacteria from different species of marine sponges from Rio de Janeiro coast (Brazil), and while assessing their resistance to different antimicrobial compounds, including trimethoprim/sulfamethoxazole, it was found that approximately one third (31%) of analyzed strains was resistant to trimethoprim/sulfamethoxazole, the highest frequency of resistance. Cultures containing ARS came in second in terms of the number of isolates obtained. Since sponges are often exposed to contamination with toxic metals and metalloids, elements like arsenic can accumulate in sponge material. Consequently their associated-bacteria tend to be resistant to high arsenic concentrations, and some exhibit the ability to bioaccumulate this element (Yamaoka *et al.*, 2001; Keren *et al.*, 2015; Keren *et al.*, 2017). This may explain that in the present work, a high number of bacteria was obtained in selective medium containing ARS.

Selective media containing CLOR, KAN, MER or TETRA produced lower numbers of isolates, which indicate a higher susceptibility of the sponge-associated bacteria to these antibiotics.

Isolated strains were assigned to different genera, including *Pseudomonas* (the most abundant), followed by *Vibrio, Bacillus, Shewanella, Exiguobacterium, Paenibacillus, Psychrobacter and Brachybacterium. Pseudomonas* are ubiquitous microorganisms, appearing often in commensal relationships or as opportunistic pathogens of fish, crustaceans and other aquatic organisms (Kefalas *et al.,* 2003; Toranzo *et al.,* 2005). The presence of pseudomonads in marine sponges is commonly reported in the literature in cultivable dependent studies and this group has been regarded as a rich source of compounds of biotechnological interest (Miki *et al.,* 1994, Jayatilake *et al.,* 1996; Bultel-Poncé *et al.,* 1999; Singh *et al.,* 2003; Romanenko *et al.,* 2005; Marinho *et al*., 2009; Keller-Costa *et al.,* 2014). Studies involving isolation of sponge-associated *Pseudomonas* sp. have revealed the production of carotenoids, phenazine alkaloids, quinolones and a psychrophilic alkaline lipase (Miki *et al.,* 1994; Bultel-Poncé *et al.,* 1999, Kiran *et al.,* 2008). Marinho *et al.* (2009) isolated several bacterial species from marine sponges from the Brazilian coast (Rio de Janeiro); including a *Pseudomonas putida* strain capable of producing antimicrobial substances, active against multidrug resistant bacteria. Zhang *et al.* (2013) isolated a *Pseudomonas stutzeri* strain from the marine sponge *Hymeniacidon perlevis* that has a high similarity with strain 48/488/MER isolated in this work. The former, produced two dehalogenases that, according to the authors, could have applications in fine chemistry and biotechnology. Pseudomonads are regularly used in bioprocesses since they grow fast with simple nutrient demand while having enormous biosynthetic capacities. Furthermore, this genus is known to include highly efficient xenobiotic degraders and by showing high tolerance to different environmental conditions (Poblete-Castro *et al.,* 2012).

Vibrios occur commonly in aquatic environments such as marine, estuarine and freshwater systems, exhibiting an enormous physiological diversity (Ceccarelli & Colwell, 2014; Romalde *et al.,* 2014). This genus also includes pathogens to aquatic animals, and vibriosis is actually one of the most economically problematic diseases affecting

aquaculture (Toranzo *et al.*, 2009). The occurrence of commensal *Vibrio* sp. in marine sponges is often reported in the literature (Hoffmann *et al.*, 2010; Hoffman *et al.*, 2012). Santavy et al. (1990) obtained a very high frequency of *Vibrio* sp. in the culturable bacterial populations associated with the marine sponge *Ceratoporella nicholsoni*, although they were poorly represented in the surrounding water in which *Acinetobacter, Micrococcus, and Moraxella* corresponded to 95% of the culturable community. Hoffman *et al.* (2012) also isolated a novel species of this genus, *Vibrio caribbeanicus*, from the marine sponge *Scleritoderma cyanea*. Bertin *et al.* (2015) detected the production of several interesting compounds by a *Vibrio harveyi* strain isolated from the marine sponge *Tectitethya cryptam*, namely spongosine, a methoxyadenosine derivative that demonstrated a variate bioactivity profile, including anti-inflammatory and vasodilatation properties. In the present work, *Vibrio* spp. were abundant among the isolates confirming and representing a promising perspective in terms of screening for new bioactive compounds.

Literature provides frequent examples of *Bacillus* sp. isolated from marine sponges and several metabolites have been detected in these strains, including anticholinesterase compounds, antimicrobial pyrroles, bacillamide C, lipopeptides and thiopeptide antibiotics (Nagai *et al.*, 2003; Pabel *et al.*, 2003; Wang, 2006; Jin *et al.*, 2011; Pandey *et al.*, 2014; Mohan *et al.*, 2016; Van Zyl *et al.*, 2016). Among all culturable bacteria isolated from marine sponges by Santos-Gandelman *et al.* (2013), *Bacillus* represented the majority. In our study, Bacilli were also well represented as well as *Brachybacterium* and *Psychrobacter* that were also reported in the study by Santos-Gandelman *et al.* (2013).

Brachybacterium sp. has been isolated from marine sources, including coastal sand, corals, deep-sea sediments, sponges and fish (Montalvo et al., 2005; Chou *et al.*, 2007; Wang *et al.*, 2009; Orsod *et al.*, 2012; Mahmoud & Kalendar, 2016). This genus is characterized by some interesting physiological features such as tolerance to extreme conditions of temperature and salinity (Junge *et al.*, 1998) and the ability to remove xenobiotics (Wang *et al.*, 2009). A sponge-associated *Brachybacterium paraconglomeratum* was isolated by Kiran *et al.* (2014) and revealed the production of a powerful glycolipid biosurfactant. Apart from this study, not many other references report the production of biologically relevant compounds in *Brachybacterium* sp. associated to

marine sponges. The association of Porifera with *Brachybacterium* as well as *Exiguobacterium*, *Psychrobacter* and *Shewanella*, often isolated from extreme environmental conditions, such as Antarctic sponges (Vishnivetskaya *et al.*, 2009; Papaleo *et al*., 2011; Rua *et al.*, 2014; Tedesco *et al.*, 2016) is worthy of further investigation. They may be regarded as extremophiles and represent an unexplored potential in terms of the compounds involved in mechanisms of physiological adaptation. For example, strains of *Exiguobacterium* grow in temperatures ranging from -12 to 55ºC under nutrient-limiting conditions and are known to produce extremozymes, such as proteases, pullulanases and lipases that show high stability properties (Kasana & Pandey, 2017). Those characteristics make this genus a potential candidate within applications in agriculture and industry.

Information on *Paenibacillus* in different environments, including animals, plants, aquatic systems and even marine hot springs, is widely available (Bouraoui *et al*., 2013; Grady *et al.*, 2016; Yang *et al*., 2016). Literature provides also examples of bacteria belonging to this genus in marine sponges such as *Erylus deficiens* and *Suberites zetequi* (Zhu *et al.*, 2008; Graça *et al.*, 2015). *Paenibacillus* strains (e.g. *Paenibacillus polymyxa* JB-0501) have been proposed as probiotics in aquaculture (Naghmouchi *et al.*, 2013; Gupta *et al*., 2016). Furthermore, this genus represents an important biotechnological potential, since *Paenibacillus* produces a broad range of antimicrobials, enzymes, and exopolysaccharides with pharmacological relevance, as well as interesting for bioremediation and process manufacturing, and several strains are already available in the market (Grady *et al.*, 2016).

The approach used in this study allowed obtaining culturable bacterial strains indicating the presence of plasmids in four of the six tested selective cultures (ARS, CLOR, TRIM and MER), which corresponded to 42.9% of total isolates. In a study by Santos-Gandelman *et al.* (2013) with marine sponges form Brazilian coastal waters, 38% of the isolates had one or more plasmid, although the isolation approach (e.g. lack of SCs and a different culture medium) and the sponge species were different.

It is important to note that in some cultures, such as CLOR and KAN, although the number of isolates were reduced, plasmids were detected in a high fraction (66.7 % in both). It is also important to mention that the highest amount of plasmid-positive strains

(46.7%) came from TRIM selective cultures. Among the tested SCs, similarly to TRIM, ARS selective cultures produced a high number of isolates; however, it produced a lower frequency of plasmid-positive strains. This may suggest that ARS selected for bacteria containing arsenic resistance features conferred by genes that were very likely in the chromosome. MER selective cultures showed the opposite trend since they produced a low number of isolates, but half of them contained plasmids. This was interpreted as an indication that the toxicity of MER inhibited the growth of many bacteria and that plasmids were important as a resistance determinant. In general, our results indicate that the addition of SC in the culture medium may be a suitable strategy to improve the chances to obtain bacterial isolates caring plasmids. However, this should be better evaluated in future studies applying experimental designs specifically planned to test this hypothesis.

Gel electrophoresis analysis of allowed to estimate the presence of plasmid DNA in the isolated bacteria. However, a limitation of this approach is the impossibility to know exactly the number of plasmids in the cells because different plasmids may not efficiently separate during the electrophoresis and bands will overlap. That means that plasmid-positive results corresponding to one or two bands may actually represent a rough underestimate. The analysis of the plasmids by advanced sequencing will be conducted as a following of this work and will allow a more detailed and complete characterization of the plasmids present in the sponge (e.g. size, variety, complete sequence).

Knowing the complete sequences of the plasmids would highlight the relation between the presence of genes with specific functions. Plasmids can be classified as conjugative, degradative, virulence, or other categories according to their role in the host cell (Harrison & Brockhurst, 2012). Plasmid functions could be investigated by searching for specific marker genes for the determination of the different families of these elements, such as the mobility (MOB) genes (e.g. oriT, relaxase gene, T4SS) which confer the ability of conjugative transfer (Shintani *et al.*, 2015). Since these isolates were obtained from selective cultures, resistance genes for the specific compound will likely be present. Multidrug resistance plasmids are common in nature and different genes associated with resistance to antibiotics and heavy metals often appear in a single plasmid or transposon (Summers, 2002; Baker-Austin *et al.,* 2006; Stepanauskas *et al*., 2006; Sandegren *et al*.,

2012). Therefore, it would be possible also to identify other resistance genes rather than those directly related with the selective pressure impose in a particular culture. By the same rationale, it is possible that similar plasmids are detected in strains from different selective cultures if the plasmids contained the resistance genes for several of the selective factors. Having the plasmid sequence available, there is also the possibility of searching for genes associated to the production of bioactive compounds and enzymes and since we used an endogenous isolation approach (culture-dependent) it is possible to obtain the plasmids *in vivo*, which represents an advantage for further applications.

Functional screening tests were also performed on the isolates in order to gain information on their ability to produce hemolysins, anionic biosurfactants and quorum quenching molecules. Blood agar is a rich culture medium that can be used to screen for hemolysins (Mulligan *et al.* 1984). Positive results correspond to the lysis of erythrocytes. This method can be used for the screening biosurfactant production, since many of these substances cause hemolysis (e.g. surfactin, rhamnolipids) (Heyd *et al.*, 2008; Walter *et al.*, 2013). Yet, it can only be considered a preliminary test for the detection of biosurfactants, since enzymes can also have hemolytic activity (e.g. lysozyme) (Youssef *et al.*, 2004; Walter *et al.*, 2013). Partial hemolysis was detected in *Vibrio*, *Psychrobacter* and *Shewanella* isolates in which plasmids were not detected which suggests that the production of haemolysins, in that case, is probably linked to chromosomal genes. Several members of *Vibrio* genus are known as serious pathogens of fish and invertebrates (e.g. *Vibrio harveyi*), and as such, have the ability to produce toxins, including hemolysins (Austin & Zhang, 2006). After using several screening methods for biosurfactants, Padmavathi *et al.* (2014), including blood agar hemolysis tests, identified coral associated bacteria belonging to the genus *Psychrobacter*, which produced thermostable biosurfactants with high anti-biofilm activity. It is possible to find several examples in the literature concerning positive hemolytic results by *Shewanella* sp., such as the marine bacteria *Shewanella algae* for which the production of hemolysins is considered an important virulence factor (Khashe *et al*., 1998; Gram *et al*., 1999; Aigle *et al*., 2015). Our results showed that all *Bacillus* isolates (*Bacillus cereus* and *Bacillus* sp.) were β-hemolytic. *B. cereus* strains are known to be β-hemolytic, due to the production of enterotoxins such as hemolysin BL (Beecher *et al.*, 1995; Beecher *et al.*, 1997). In previous works, other species

of *Bacillus*, such as *Bacillus thuringiensis* and *Bacillus subtilis* were also already identified as producers of hemolysins (Budarina *et al.*, 1994; Pan *et al.*, 2014). We also obtained a strain of *Pseudomonas aeruginosa* showing β-hemolytic activity and that was plasmid positive. *P. aeruginosa* strains are described as being mostly hemolytic (Iglewski, 1996) and their associated hemolysins (e.g. Heat-stable hemolysin, Phospholipase C) have an important role in its pathogenesis (Barker *et al.,* 2004; Soberón-Cháves *et al.*, 2005). Previous findings have already detected plasmids bearing genes coding for the production of hemolysins in marine bacteria, such as *Vibrio parahemolyticus* (Nishibuchi & Kaper, 1995). Although we did not establish a relation between hemolytic activity and the presence of plasmid DNA in this study, a further analysis of plasmid DNA sequences can bring a decisive perspective into this topic.

In the present work, the MB-CTAB agar plate method was used as a semi-quantitative assay for the detection of extracellular rhamnolipids as well as other anionic biosurfactants (Siegmund & Wagner, 1991; Pinzon & Ju, 2009) but no evidence of biosurfactant production was found in any of the isolates. However, it is still possible that some of our strains may still synthetize other types of surfactants. Therefore, other screening tests (e.g. drop collapse, oil spray, emulsification index) will be performed as a continuation of the work. Biosurfactants appear as an interesting alternative to chemical surfactants, due to their low toxicity and high biodegradability. Marine environments are frequently screened for biosurfactant producing bacteria (Thavasi *et al*., 2013; Elazzazi *et al.,* 2015; Hu *et al*., 2015). Dhasayan *et al*. (2014) isolated a biosurfactant producing strain of *Bacillus amyloliquefaciens* from the marine sponge *Callyspongia diffusa*. 14.3% of the sponge isolates obtained in this study belonged to the Bacillus genus. Also some biosurfactant-producing strains have been isolated in remote and extreme microhabitats and the corresponding molecules have unsurprisingly higher stability in extreme physicochemical conditions (e.g. temperature, pH, salinity) and are promising for application in industrial processes (Geys *et al.,* 2014).

Quorum sensing is a mechanism of cell-to-cell communication involving chemical signaling molecules that regulates gene expression on populations of microbial cells. This process in involved in regulation of cell density, virulence, biofilm formation (Rutherford

& Bassler, 2012). Bacterial biofilms are associated with environmental processes such as biofouling and biocorrosion, but also in numerous chronic diseases, since microbial biofilms are less susceptible to the traditional antibiotic treatment (McLean *et al.*, 2004; Beech *et al.*, 2005; Taraszkiewicz *et al.*, 2013). Therefore, the inhibition of quorum sensing is actually regarded as a key factor in the control of microbial infections. Many organisms have the ability to produce quorum quenching or quorum-sensing inhibiting molecules (e.g. acylases, lactonases, paraoxonases) which limit cell growth and prevent the appearing of pathogenic phenotypes (Beech *et al.*, 2005; Chen *et al.*, 2013). In this study, positive/strong positive quorum quenching was more frequently detected in *Pseudomonas* spp. Curiously, the great majority of the Pseudomonads positive for quorum quenching were also carrying plasmids. A previous study showed that quorum quenching molecules were detected in *Pseudomonas aeruginosa* PAO1, where the chromosomal gene PA2385 encodes for a N-Acyl-Homoserine Lactone acylase that prevents quorum-sensing systems dependent on N-acyl-Homoserine lactone molecules from functioning (Sio *et al.*, 2006). In this work, the effect of quorum sensing inhibition was also observed in *Bacillus* and *Vibrio* isolates. Saurav *et al.* (2016) investigated the production of quorum quenchers by bacterial symbionts of marine sponges and obtained positive results in *Bacillus* sp. and *Vibrio* sp. isolates. Recent research has demonstrated that bacterial symbiont communities in marine sponges cooperate through quorum sensing, being N-acyl-homoserine lactones (NAHLs) the most studied signaling molecule in this context (Mohamed *et al.*, 2008; Gàrderes *et al.*, 2012; Britstein *et al.*, 2015). Skindersoe *et al.* (2008) screened for quorum sensing molecules in several marine organisms obtained from the Great Coral Reef, concluded that sponges were amongst the most frequent producers of quorum quenching active extracts. It has been suggested that the symbionts of sponges may compete with other bacteria through the blockage of inter-cell communication. Deepening the study of these processes may allow not only the discovery of new quorum quenching compounds, but also a better understanding of the ecology of the sponge holobiont.

Concluding Remarks

5- Concluding Remarks

Information on the plasmid hosts in sponge bacterial symbionts is particularly scarce and this work represents one of the few attempts to isolate plasmid-carrying bacteria from marine sponges. Our results showed that members of the *Pseudomonas* genus were the most common culturable plasmid hosts in the studied sponges. Curiously, among 14 *Pseudomonas* strains carrying plasmids, only 3 failed to show quorum quenching activity. However, this pattern does not provide any explanation for the causal relation between plasmids and quorum quenching activity in the *Pseudomonas* strains isolated in this study. Further studies are necessary to evaluate if the genes encoding for quorum-quenchers could be located in plasmids. In contrast to these results, the production of hemolysins was mainly associated with plasmid-negative strains, which suggests that genes encoding for these molecules are chromosomal. In future works, we expect to further evaluate the potential of the isolates and their plasmids for the production of enzymes and metabolites of biotechnological potential. Considering the data acquired from functional screening tests we intend to sequence the plasmids obtained and search for genes encoding for metabolic pathways involved in the degradation of specific molecules, such as N-Acyl homoserine lactones and for other families of bioactive compounds of biotechnological interest.

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Supporting Information

8- Supporting Information

Table S1- *Sampling localities and coordinates of the different species of marine sponges used in this study.*

Sample	Sponge species	Location (Taiwan)	GPS coordinates
PSC 394	*Cinachyrella sp.*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E
PCS 395	*Cinachyrella sp.*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E
PSC 397	*Cinachyrella sp.*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E
PSC 410	*Xestospongia testudinaria*	Dongji island	23°15'27.17"N, 119°40'45.28"E
PSC 446	*Ptilocaulis sp.*	Siji Island	23°14'35.80"N, 119°36'53.56"E
PSC 448	*Ptilocaulis sp.*	Siji Island	23°14'35.80"N, 119°36'53.56"E
PSC 449	*Ptilocaulis sp.*	Siji Island	23°14'35.80"N, 119°36'53.56"E
PSC 486	*Xestospongia testudinaria*	Xiyu Island	23°34'21.58"N, 119°29'35.14"E
PSC 488	*Xestospongia testudinaria*	Xiyu Island	23°34'21.58"N, 119°29'35.14"E
PSC 492	*Xestospongia testudinaria*	Xiyu Island	23°34'21.58"N, 119°29'35.14"E
PSC 522	*Suberites sp.*	Maoyu Islet	23°19'27.62"N, 119°19'20.37"E
PSC 523	*Suberites sp.*	Maoyu Islet	23°19'27.62"N, 119°19'20.37"E
PSC 524	*Suberites sp.*	Maoyu Islet	23°19'27.62"N, 119°19'20.37"E
PSC 525	*Suberites sp.*	Maoyu Islet	23°19'27.62"N, 119°19'20.37"E
PSC 526	*Agelas cavernosa*	Maoyu Islet	23°19'27.62"N, 119°19'20.37"E
PSC 565	*Xestospongia testudinaria*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E
PSC 568	*Xestospongia testudinaria*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E
PSC 571	*Cinachyrella sp. or Paratetilla sp.*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E
PSC 572	*Cinachyrella sp. or Paratetilla sp.*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E
PSC 575	*Cinachyrella sp.*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E
PSC 576	*Cinachyrella sp.*	Aimen Beach	23°33'2.54"N, 119°38'26.83"E

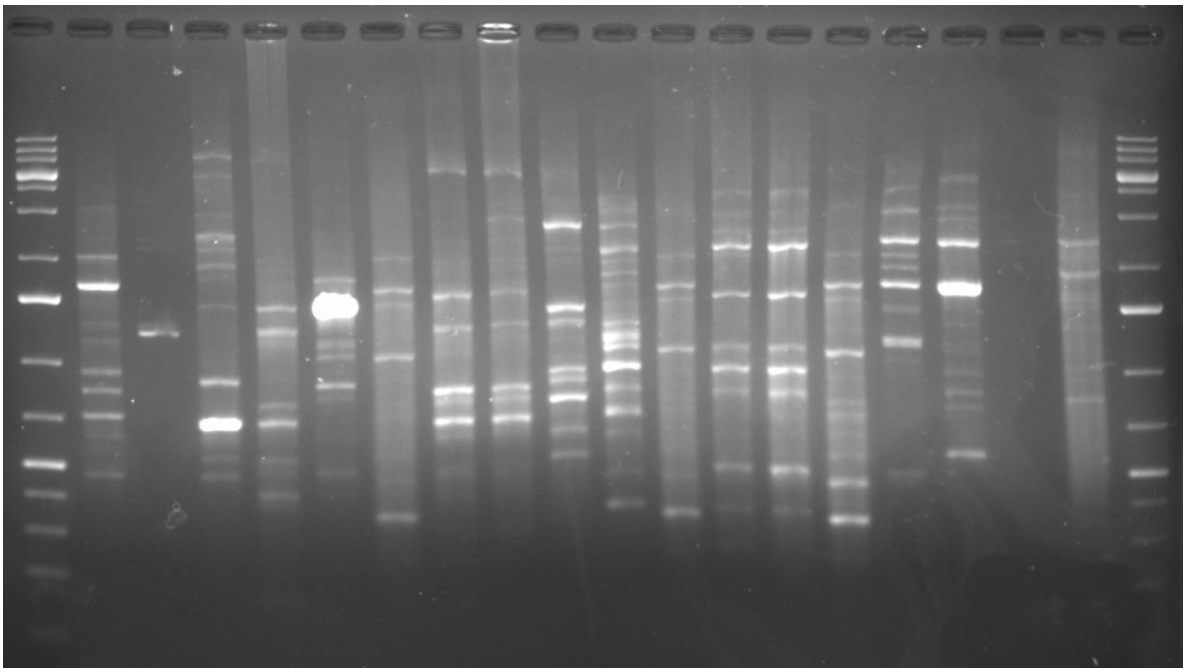

Figure S1 *- Agarose electrophoresis of Box-PCR genomic fingerprint patterns of the bacteria isolated from marine sponges. 1) M (DNA ladder, Gene Ruler 1Kb Plus), 2) 24/394/ARS, 3) 24-1/394/ARS, 4) 72-1/410/ARS, 5) 24/449/ARS, 6) 48/522/ARS, 7) 24/523//ARS, 8)24-1/524/ARS, 9) 24-2/524/ARS, 10) 24-1/526/ARS, 11) 72/526/ARS, 12) 24/565/ARS, 13) 24/568/ARS, 14) 24/572/ARS, 15) 48/572/ARS, 16) 48/394/MER, 17) 48/395/MER, 18) Negative Control 19) 48/488/MER, 20) M.*

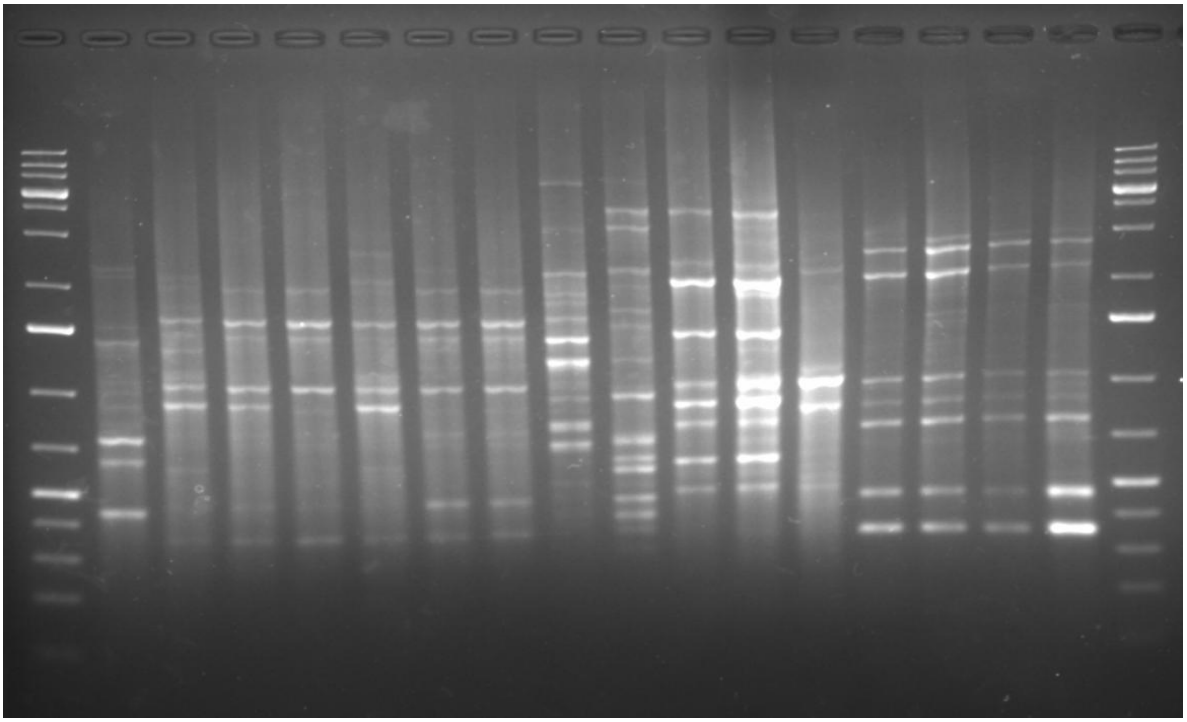

Figure S2 *- Agarose electrophoresis of Box-PCR genomic fingerprint patterns of the bacteria isolated from marine sponges. 1) M, 2) 72-2/394/CLOR, 3) 72/448/CLOR, 4) 48/523/CLOR, 5)24/525/CLOR, 6) 48/526/CLOR, 7) 48/565/CLOR, 8) 24-1/572/CLOR, 9) 24/486/KAN, 10) 48/522/KAN, 11) 48/526/KAN, 12) 24-2/526/ARS, 13) 72-1/397/TETRA, 14) 24/571/TETRA, 15) 24/571/TRIM, 16) 48/571/TETRA, 17) 24/571/ARS, 18) M.*

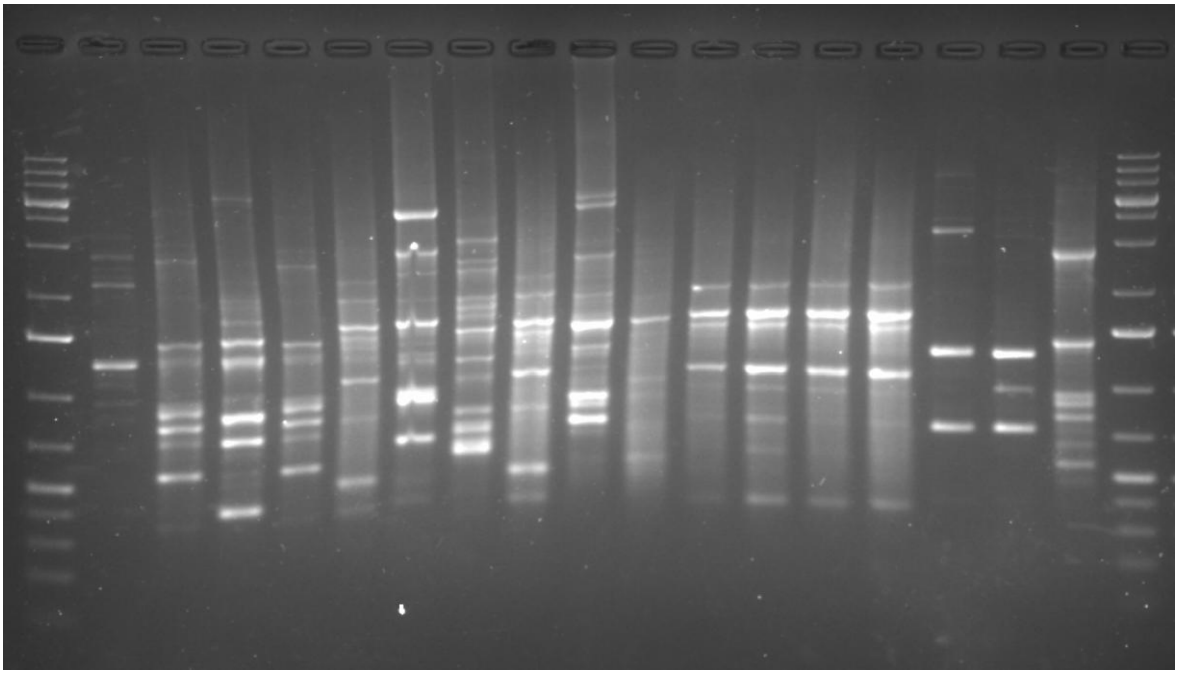

Figure S3 *- Agarose electrophoresis of Box-PCR genomic fingerprint patterns of the bacteria isolated from marine sponges. 1) M, 2) 24/394/TRIM, 3) 24-1/446/TRIM, 4) 24-2/446/TRIM, 5) 72-2/448/TRIM, 6) 72/446/TRIM, 7) 24/449/TRIM, 8) 24/492/TRIM, 9) 24/523/TRIM, 10) 24/524/TRIM, 11) 48/525/TRIM, 12) 24/526/TRIM, 13) 24/565/TRIM, 14) 48/568/TRIM, 15) 48/572/TRIM, 16) 24/575/TRIM, 17) 24/476/TRIM, 18) 48/449/MER, 19) M.*

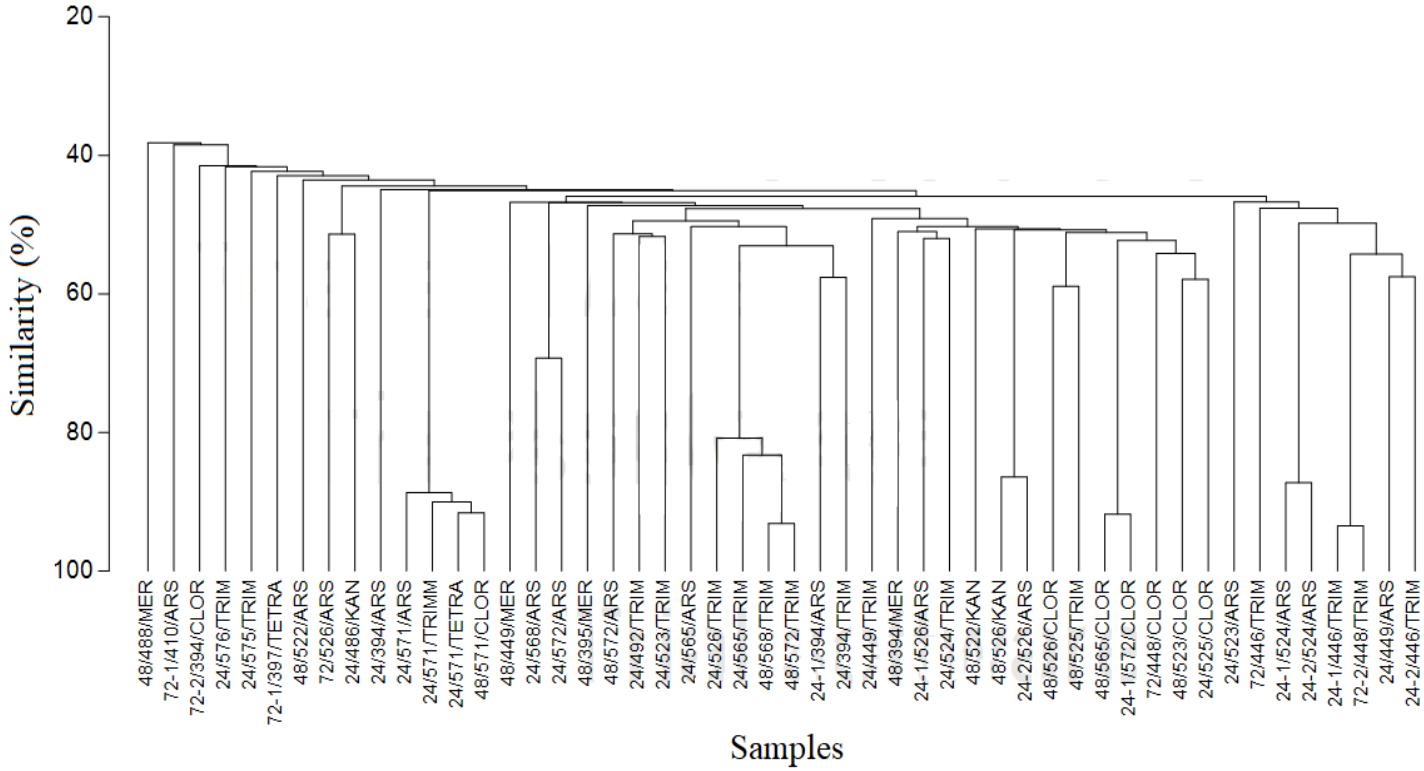

Figure S4- *Clustered data obtained from Box PCR genomic fingerprints of the bacteria isolated from marine sponges. Investigated bacterial isolates were identified as DD/PSC/SC (e.g. 24/565/ARS), where DD represents the time of growth in TSB (e.g. 24 h); PSC reflects the code of the sample (e.g. PSC 565) and SC the selective compound added to the medium (e.g. ARS).*